

CANINE RESPIRATORY CORONAVIRUS (CRCV)
SPIKE PROTEIN, POLYMERASE AND
HEMAGGLUTININ/ESTERASE

The present invention relates to biological material, and in particular to a canine respiratory coronavirus that is present in dogs having canine infectious respiratory disease.

- 5 Canine infectious respiratory disease (CIRD) is a highly contagious disease common in dogs housed in crowded conditions such as re-homing centres and boarding or training kennels. Many dogs suffer only from a mild cough and recover after a short time, however in some cases a severe bronchopneumonia can develop (Appel and Binn, 1987).
- 10 The pathogenesis of CIRD is considered to be multifactorial, involving several viruses and bacteria. The infectious agents considered to be the major causative pathogens of CIRD are canine parainfluenzavirus (CPIV) (Binn *et al.*, 1967), canine adenovirus type 2 (CAV-2) (Ditchfield *et al.*, 1962) and the bacterium *Bordetella bronchiseptica* (Bemis *et al.*, 1977, Keil
- 15 *et al.*, 1998). Also, canine herpesvirus, human reovirus and mycoplasma species have been isolated from dogs with symptoms of CIRD (Karpas *et al.*, 1968, Lou and Wenner 1963, Randolph *et al.*, 1993) Additional factors like stress may also be important.

CIRD is rarely fatal but it delays re-homing of dogs at rescue centres and it

20 causes disruption of schedules in training kennels as well as considerable treatment costs.

Vaccines are available against some of the infectious agents associated with this disease, namely *Bordetella bronchiseptica* as well as CPIV and CAV-2. However, despite the use of these vaccines, CIRD is still prevalent in

25 kennels world-wide, which is possibly due to the vaccines not providing protection against all the infectious agents involved in CIRD.

We have discovered a novel coronavirus, which we have called canine respiratory coronavirus (CRCV), in a large kennelled dog population with a history of endemic respiratory disease, and we have shown that this virus is associated with CIRD.

- 5 Some members of the family *coronaviridae* are known to cause respiratory disease in humans, cattle, swine and poultry (Mäkelä *et al.*, 1998, Pensaert *et al.*, 1986, Ignjatovic and Sapats 2000). For example, bovine respiratory coronavirus is associated with shipping fever in cattle which is a multifactorial respiratory disease (Storz *et al.*, 2000).
- 10 However, coronaviruses were not suspected to have a role in the pathogenesis of CIRD. Indeed, with only a single exception, canine coronaviruses have been reported to be enteric viruses and to cause acute diarrhoea mainly in young dogs (for example, Tennant *et al.*, 1993). In a large study of viruses involved in canine respiratory diseases, Binn *et al.*
- 15 (1979) reported the detection of a canine coronavirus in the lung of a single dog that was also infected with SV5 and canine adenovirus 2, two other viruses that are associated with canine respiratory disease.

- There are 30-40 dog vaccines commercially available in the UK for use against a number of pathogens that can cause a range of diseases, such as
- 20 neurological, enteric, hepatic and respiratory diseases. Most of these vaccines contain microbial agents such as Distemper virus, Canine Adenovirus-2, Canine parvovirus, canine parainfluenza virus and *Leptospira canicola* and *L. icterohaemorrhagiae*. None of these vaccines contain canine coronaviruses.

- 25 The dog vaccines for use against canine respiratory diseases are marketed as vaccines for "kennel-cough" (see below). All of the vaccines contain

Bordetella bronchisepticum, which is a bacterium associated with “kennel cough”.

Coyne M.J. & May S.W., (1995) in their article entitled “Considerations in using a canine coronavirus vaccine” (published as a Pfizer Technical Bulletin on the Internet at <http://www.pfizer.com/ah/vet/tref/trbull/ccv.html>), lists over 20 commercially available vaccines against either canine coronaviruses alone or against canine coronaviruses together with other organisms. Each of these vaccines is for canine enteric disease, and there is no suggestion that a canine coronavirus may be associated with respiratory disease.

US Patents Nos. 6,057,436 and 6,372,224, both to Miller *et al* and assigned to Pfizer, Inc., describe the spike gene of the enteric canine coronavirus and uses therefor, including its use as a vaccine against gastroenteritis. Neither of these two patents suggest that a canine coronavirus may be involved in CIRD.

Members of the family *coronaviridae* are enveloped viruses, 80-160nm in diameter, containing a linear positive-stranded RNA genome. The structural proteins of coronaviruses are the spike glycoprotein (S), the membrane glycoprotein (M) and the nucleocapsid protein (N). The hemagglutinin/esterase glycoprotein (HE) is found only on the surface of group II coronaviruses (e.g. bovine coronavirus and murine hepatitis virus) (Spaan *et al*, 1988). Further details of the structure of coronaviruses may be found in the chapter by Cavanagh *et al* entitled “Coronaviridae” p407-411, in “Virus Taxonomy, 6th Report of the International Committee on Taxonomy of Viruses”, pub. Springer-Verlag Wein, New York, Eds. Murphy *et al*, which is incorporated herein by reference.

The canine respiratory coronavirus (CRCV) of the invention may be characterised as a coronavirus present in the respiratory tracts of dogs with infectious respiratory disease. To further characterise CRCV, we have determined the sequence of 250 nucleotide residues of the CRCV polymerase (pol) cDNA (Figure 1 and SEQ ID NO: 1) which corresponds to an 83 amino acid partial sequence of the pol protein (Figure 2 and SEQ ID NO: 2). We have also cloned and determined the sequence of the 4092 nucleotide residues of the CRCV spike (S) cDNA (Figure 3 and SEQ ID NO: 3), corresponding to 1363 amino acids (Figure 4 and SEQ ID NO: 4). We have also determined the sequence of 497 nucleotide residues of the CRCV hemagglutinin/esterase (HE) gene (Figure 13 and SEQ ID NO: 21), corresponding to 165 amino acids (Figure 14 and SEQ ID NO: 22). We have identified that CRCV has a surprisingly low homology to the enteric canine coronavirus (CCV) while it has an unexpectedly high level of homology to bovine coronavirus (strain LY138 or Quebec) and human coronavirus (strain OC43).

A culture of "Spike D-1 CRCV", which is XL1-Blue *E. coli* (Stratagene) containing a pT7Blue2 plasmid (Novagen) whose insert contains a portion of the CRCV spike cDNA, has been deposited under the Budapest Treaty at NCIMB Ltd under Accession number NCIMB 41146 on 25 July 2002. The depositor of NCIMB 41146 is the Royal Veterinary College, Royal College Street, London NW1 OTU, UK. The address of NCIMB Ltd is 23 St. Machar Drive, Aberdeen, Scotland, AB24 3RY, UK.

The phylogenetic relationship of CRCV to eleven known coronaviruses was determined based upon a comparison of the 250 nucleotide sequence from the CRCV pol gene and the corresponding regions of the other viruses (Figure 5). The bovine coronavirus (BCV), human coronavirus (HCV) strain OC43 and hemagglutinating encephalomyelitis virus (HEV) were

found to be most closely related to CRCV, while the enteric CCV was found to be only distantly related to CRCV.

Over the 250 sequenced residues of the pol cDNA, corresponding to 83 amino acids, CRCV has only 68.5% and 75.9% sequence identity at the nucleotide and amino acid levels, respectively, with the equivalent region of the enteric CCV (strain 1-71) pol gene (Genbank Accession No. AF124986), as shown in Figure 6 and 7.

Over the 4092 sequenced nucleotide residues of the CRCV S gene, corresponding to 1363 amino acids, CRCV has 45% and 21.2% sequence identity at the nucleotide (Figure 8) and amino acid levels, respectively, with the equivalent region of the enteric CCV (strain 1-71) S gene.

Enteric CCV is not a group II coronavirus and does not possess an HE gene, hence it is not possible to determine the extent of sequence identity between this gene in CRCV and in enteric CCV.

Except as described below, the percentage identity between two nucleotide or two amino acid sequences was determined using FASTA version 34 (Pearson WR. (1990) "Rapid and sensitive sequence comparison with FASTP and FASTA". *Methods Enzymol.*;183:63-98). FASTA settings were Gap open penalty -16 and Gap extension penalty -4.

The percentage identity between the CRCV and enteric CCV spike sequences was determined using GCG version 10 (Genetics Computer Group, (1991), Program Manual for the GCG Package, Version 7, April 1991, 575 Science Drive, Madison, Wisconsin, USA 53711). The GCG parameters used were: Gap creation penalty 50, gap extension penalty 3 for DNA, and Gap creation penalty 8 and Gap extension penalty 2 for Protein.

Sequence alignments were performed using ClustalX (Thompson *et al.*, 1997).

By contrast, over the 250 sequenced residues of the pol cDNA, CRCV has 98.8% sequence identity with the equivalent region of the BCV strain Quebec pol gene (Genbank Accession No. AF220295), 98.4% sequence
5 identity with the BCV strain LY138 pol gene (Genbank Accession No. AF124985) and 98.4% sequence identity with the HCV OC43 pol gene (Genbank Accession No. AF124989).

There was only a single amino acid difference between the CRCV pol
10 protein over the 83 sequenced amino acids and the BCV, HCV and HEV pol proteins which is that CRCV has E (Glu) as opposed to D (Asp) at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29). Thus the CRCV pol protein is 99% identical to the BCV, HCV and HEV pol proteins over this region.

15 The one and three letter amino acid codes of the IUPAC-IUB Biochemical Nomenclature Commission are used herein.

Over the 497 sequenced nucleotide residues, corresponding to 165 amino acids, of the HE gene, CRCV has 98.994% and 98.2% sequence identity with the equivalent region of the BCV strain LY138 HE gene (Genbank
20 Accession No. AF058942) at the nucleotide and amino acid levels respectively. CRCV has 98.189% (nucleotide) and 98.2% (amino acid) sequence identity with human enteric coronavirus (HECV) HE gene (Genbank Accession No. L07747); 97.4% (nucleotide) and 95.2% (amino acid) sequence identity with the HCV OC43 HE gene (Genbank Accession
25 No. M76373); and 92.0% (nucleotide) and 93.9% (amino acid) identity with HEV (Genbank Accession Nos. AF481863), as shown in Figures 15 and 16.

As shown in Figure 16 and Table 3, the three amino acids that are different between the CRCV HE protein and each of the BCV, HECV, HCV and HEV S proteins, within the 165 amino acids of the CRCV HE protein, are F (Phe) as opposed to L (Leu), N (Asn) as opposed to T (Thr), and L (Leu) as opposed to V (Val) at positions corresponding to position 235, 242 and 253, respectively, in the BCV, HECV, HCV OC43 and HEV HE genes (Figure 16). Thus F at position 235, N at position 242 and L at position 253 could be said to be CRCV HE protein-specific amino acids.

Over the 4092 sequenced nucleotide residues, corresponding to 1363 amino acids, of the CRCV S gene, CRCV has 97.3% and 96% identity with the equivalent region of BCV strain LY138 (Genbank Accession No. AF058942) at the nucleotide and amino acid levels respectively. CRCV has 96.9% (nucleotide) and 95.2% (amino acid) identity with HCV strain OC43 (Genbank Accession No. Z32768), and 83.8% (nucleotide) and 80.4% (amino acid) identity with HEV (Genbank Accession Nos. AF481863 (cDNA) and AAM 77000 (protein)) as shown in Figures 9 and 10.

The amino acids that are different between the CRCV S protein and each of the BCV, HCV and HEV S proteins, within the 1363 amino acids of the CRCV S protein, are listed in Table 1 below. Thus the amino acids listed in Table 1 could be said to be CRCV S protein-specific amino acids. The amino acids are numbered from the initial M residue at the start of the CRCV protein, as shown in Figure 4.

Table 1: List of 39 amino acids specific to the CRCV S protein that are not present in the BCV, HCV and HEV S proteins.

<u>Position</u>	<u>Amino acid</u>
103	V
118	V
166	D
171	M
179	K
192	P
210	S
235	H
267	F
388	F
407	M
436	S
440	I
447	I
501	F
525	Y
528	N
540	L
582	K
608	G

<u>Position</u>	<u>Amino acid</u>
692	G
695	S
757	W
758	G
763	Q
769	T
786	P
792	H
818	R
827	P
828	V
887	F
933	D
977	F
1011	T
1018	S
1063	K
1256	L
1257	M

A first aspect of the invention provides a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with the CRCV S protein whose amino acid sequence is listed in Figure 4, and having at least one of V at position 103; V at position 118; D at position 166; M at position 171; K at position 179; P at position 192; S at position 210; H at position 235; F at position 267; F at position 388; M at position 407; S at position 436; I at position 440; I at position 447; F at position 501; Y at position 525; N at position 528; L at position 540; K at position 582; G at position 608; G at position 692; S at position 695; W at position 757; G at position 758; Q at position 763; T at position 769; P at position 786; H at position 792; R at position 818; P at position 827; V at position 828; F at position 887; D at position 933; F at position 977; T at position 1011; S at position 1018; K at position 1063; L at position 1256; and M at position 1257. The amino acids are numbered from the initial M at the start of the CRCV S protein, as listed in Figure 4 (SEQ ID NO: 4).

It is appreciated that the partial nucleotide sequence of CRCV S can be readily determined by a person of ordinary skill in the art by sequencing the insert of the plasmid contained in *E. coli* strain D-1 CRCV, that has been deposited under the Budapest Treaty at NCIMB Ltd. under Accession number NCIMB 41146 on 25 July 2002. Furthermore, this DNA can be used as a hybridisation probe, or as the basis for the design of probes, in the isolation of CRCV nucleic acid in dogs.

For the avoidance of doubt, the invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with the CRCV S protein (SEQ ID NO: 4), and comprising at least one of the amino acids specific for the CRCV S protein at the position listed in Table 1.

By "protein" we also include the meaning glycoprotein. The amino acid sequence of a glycoprotein refers to the amino acid sequence of the polypeptide backbone of the glycoprotein, irrespective of the type, number, sequence and position of the sugars attached thereto.

- 5 Typically, the invention includes an isolated or recombinant protein, and not an unmodified CRCV protein present as a CRCV component.

The invention includes a coronavirus S protein, or fragment thereof, having at least 76% amino acid sequence identity with the CRCV S protein (SEQ ID NO: 4), or at least 77%, or at least 78%, or at least 79%, or at least 80%,
10 or at least 81%, or at least 82%, or at least 83%, or at least 84%, or at least 85%, or at least 86%, or at least 87%, or at least 88%, or at least 89%, or at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with the CRCV S protein, and
15 comprising at least one of the amino acids specific for the CRCV S protein at the position listed in Table 1.

The invention also includes a coronavirus S protein, or fragment thereof, having at least 75%, or at least 80%, or at least 85% or at least 90% or at least 95% amino acid sequence identity with the CRCV S protein (SEQ ID
20 NO: 4), and comprising at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 11, or at least 12, or at least 13, or at least 14, or at least 15, or at least 16, or at least 17, or at least 18, or at least 19, or at least 20, or at least 21, or at least 22, or at least 23, or at least 24, or at least 25, or at least 26, or at least 27, or
25 at least 28, or at least 29, or at least 30, or at least 31, or at least 32, or at least 33, or at least 34, or at least 35, or at least 36, or at least 37, or at least 38 of the amino acids specific for CRCV S protein at the positions listed in Table 1.

Preferably, the coronavirus S protein, or fragment thereof comprises all 39 of the amino acid residues specific for CRCV S protein at the positions listed in Table 1.

Thus the invention includes a BCV, HCV or HEV S protein or fragment thereof, that has been modified at at least one position listed in Table 1 to resemble the CRCV S protein.

Preferably, the coronavirus S protein of the invention is a CRCV S protein that comprises or consists of the sequence listed in Figure 4 (SEQ ID NO: 4), or a variant thereof with at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

Thus the variant of the coronavirus S protein of the invention includes a protein that comprises or consists of the sequence listed in Figure 4 (SEQ ID NO: 4) but has between 1 and 40 amino acid differences from the sequence listed in Figure 4. Preferably, the variant has less than 40 amino acid differences from the sequence listed in Figure 4. More preferably the variant has less than 35, less than 30, or less than 25, or less than 20, or less than 15, or 10 or 9 or 8 or 7 or 6 or 5 or 4 or 3 or 2 amino acid differences, or a single amino acid difference, from the sequence listed in Figure 4.

The invention also includes a CRCV S protein fragment comprising a fragment of the sequence listed in Figure 4 (SEQ ID NO: 4) which comprises at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

The invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14, Genbank Accession No. AF058942), and comprising at least one of V at position 103; V at position 118; D at position 166; M at position 171; K at position 179; P at position 192; S at position 210; H at position 235; F at position 267; F at position 388; M at position 407; S at position 436; I at position 440; I at position 447; ; F at position 501; Y at position 525; N at position 528; L at position 540; K at position 582; G at position 608; G at position 692; S at position 695; W at position 757; G at position 758; Q at position 763; T at position 769; P at position 786; H at position 792; R at position 818; P at position 827; V at position 828; F at position 887; D at position 933; F at position 977; T at position 1011; S at position 1018; K at position 1063; L at position 1256 and M at position 1257.

For the avoidance of doubt, the invention includes a coronavirus S protein, or fragment thereof, having at least 75% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14), and comprising at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

The invention includes a coronavirus S protein, or fragment thereof, having at least 76% amino acid sequence identity with BCV strain LY138 S protein, or at least 77%, or at least 78%, or at least 79%, or at least 80%, or at least 81%, or at least 82%, or at least 83%, or at least 84%, or at least 85%, or at least 86%, or at least 87%, or at least 88%, or at least 89%, or at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 S protein, and

having at least one of the amino acids specific for CRCV S protein at the position listed in Table 1.

The invention also includes a coronavirus S protein, or fragment thereof, having at least 75%, or at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid sequence identity with BCV strain LY138 S protein (SEQ ID NO: 14), and comprising at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 11, or at least 12, or at least 13, or at least 14, or at least 15, or at least 16, or at least 17, or at least 18, or at least 19, or at least 20, or at least 21, or at least 22, or at least 23, or at least 24, or at least 25, or at least 26, or at least 27, or at least 28, or at least 29, or at least 30, or at least 31, or at least 32, or at least 33, or at least 34, or at least 35, or at least 36, or at least 37, or at least 38 of the amino acids specific for CRCV S protein at the positions listed in Table 1.

Preferably, the coronavirus S protein, or fragment thereof comprises all 39 of the amino acid residues specific for CRCV S protein at the positions listed in Table 1.

A second aspect of the invention provides a coronavirus pol protein, or fragment thereof, having at least 90% amino acid sequence identity with the BCV pol protein (SEQ ID NO: 5) and comprising the amino acid E at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29).

The invention includes a coronavirus pol protein, or fragment thereof, having at least 91% amino acid sequence identity with BCV strain LY138 pol protein, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 pol protein, and having the amino

acid E at the position corresponding to position 4975 in the BCV genome (Accession No. SWALL: Q91A29).

Preferably, the coronavirus pol protein, or fragment thereof is a CRCV pol protein or fragment thereof that comprises or consists of the amino acid
5 sequence listed in Figure 2.

Thus the invention includes a BCV, HCV or HEV pol protein or fragment thereof, that has been modified at the amino acid corresponding to position 4975 in the BCV genome, to resemble the CRCV pol protein.

The invention also includes a CRCV pol protein fragment comprising a
10 fragment of the sequence listed in Figure 2 (SEQ ID NO: 2) and having the amino acid E at the position corresponding to position 4975 in the BCV genome.

A third aspect of the invention provides a coronavirus HE protein, or fragment thereof, having at least 90% amino acid sequence identity with the
15 BCV LY138 HE protein (Genbank Accession No. AF058942), and having at least one of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention includes a coronavirus HE protein, or fragment thereof,
20 having at least 91% amino acid sequence identity with BCV strain LY138 HE protein, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having at least one of F at position 235; N at position 242; and L at position 253. The
25 amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention also includes a coronavirus HE protein, or fragment thereof, having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having two of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

The invention further includes a coronavirus HE protein, or fragment thereof, having at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid sequence identity with BCV strain LY138 HE protein, and having all three of F at position 235; N at position 242; and L at position 253. The amino acid positions are numbered from the initial M (which is number 1) at the start of the BCV HE protein.

Preferably, the coronavirus HE protein, or fragment thereof is a CRCV HE protein or fragment thereof that comprises or consists of the amino acid sequence listed in Figure 14 (SEQ ID NO: 22).

Thus the invention includes a BCV, HCV, HECV or HEV HE protein or fragment thereof, that has been modified at one or more of the amino acids corresponding to position 235, 242; and 253 to resemble the CRCV HE protein.

The invention also includes a CRCV HE protein fragment comprising a fragment of the sequence listed in Figure 14 (SEQ ID NO: 22) and having one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in

which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

The coronavirus S, pol and HE proteins as defined above in the first, second and third aspects of the invention may be termed herein “CRCV” or
5 “CRCV-like” proteins.

A “CRCV S protein” is an S protein or fragment thereof that has the native CRCV S amino acid sequence as listed in Figure 4 (SEQ ID NO: 4), or a fragment thereof which comprises at least one of the amino acids specific for a CRCV S protein at the positions listed in Table 1.

10 A “CRCV pol protein” is a pol protein or fragment thereof that has the native CRCV pol amino acid sequence as listed in Figure 2 (SEQ ID NO: 2), or a fragment thereof which comprises the amino acid E at the position corresponding to position 4975 in the BCV genome.

A “CRCV HE protein” is an HE protein or fragment thereof that has the
15 native CRCV HE amino acid sequence as listed in Figure 14 (SEQ ID NO: 22), or a fragment thereof which comprises one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in which residue number 1 is the
20 initial M at the start of the BCV LY138 HE protein.

A “CRCV-like S protein” is an S protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV S amino acid sequence (Figure 4 and SEQ ID NO: 4), but has at least 75% sequence identity with the corresponding region of the CRCV or BCV strain LY138 S
25 protein, and has at least one of the amino acids specific for a CRCV S protein at the positions listed in Table 1.

A "CRCV-like S protein" also includes an S protein that does not have an amino acid sequence identical to the native CRCV S amino acid sequence (Figure 4 and SEQ ID NO: 4), but that comprises or consists of a variant of the sequence listed in Figure 4 with at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

A "CRCV-like pol protein" is a pol protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV pol amino acid sequence, but has at least 90% sequence identity with the corresponding BCV strain LY138 pol protein, and which has an E at the position corresponding to position 4975 in the BCV genome.

A "CRCV-like HE protein" is an HE protein or fragment thereof that does not have an amino acid sequence identical to the native CRCV HE amino acid sequence, but has at least 90% sequence identity with the corresponding BCV strain LY138 HE protein, and which has one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these three amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

Preferably, the CRCV or CRCV-like protein, or fragment thereof, is at least 10 amino acids in length. More preferably, the CRCV or CRCV-like protein, or fragment thereof, is at least 20, or at least 30, or at least 40, or at least 50, or at least 100, or at least 200, or at least 300, or at least 400, or at

least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1,000, or at least 1,100, or at least 1,200 amino acids in length.

Preferably, the CRCV or CRCV-like protein, or fragment thereof, is less than about 1,300 amino acids in length. More preferably, the CRCV or
5 CRCV-like protein, or fragment thereof, is less than about 1,200, or less than about 1,100, or less than about 1,000, or less than about 900, or less than about 800, or less than about 700, or less than about 600, or less than about 500, or less than about 400, or less than about 300, or less than about 200, or less than about 100, or less than about 50 amino acids in length.

10 CRCV proteins may be isolated from CRCV, or may be made using protein chemistry techniques for example using partial proteolysis of isolated proteins (either exolytically or endolytically), or by *de novo* synthesis. Alternatively, the CRCV proteins, as well as CRCV-like proteins, may be made by recombinant DNA technology. Suitable techniques for cloning,
15 manipulation, modification and expression of nucleic acids, and purification of expressed proteins, are well known in the art and are described for example in Sambrook *et al* (2001) "*Molecular Cloning, a Laboratory Manual*", 3rd edition, Sambrook *et al* (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, incorporated herein by
20 reference.

Shorter fragments of CRCV and CRCV-like proteins, *ie* peptides, may be synthesised using standard techniques. Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* 46, 3433 and references therein. Temporary N-amino
25 group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine

threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where
5 glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethylacrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and
10 acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-
15 carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic
20 acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a
25 simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-
30 exchange chromatography and (principally) reverse-phase high performance

liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

- 5 A fourth aspect of the invention provides a polynucleotide that encodes a CRCV or CRCV-like S, pol or HE protein according to the first, second and third aspects of the invention, or the complement thereof.

Preferably, the polynucleotide encodes a CRCV S protein according to the first aspect of the invention, or the complement thereof.

- 10 More preferably, the polynucleotide encoding the CRCV S protein comprises or consists of the sequence listed in Figure 3 (SEQ ID NO: 3).

It is appreciated that the sequence listed in Figure 3 (SEQ ID NO: 3) contains a Y at position 3531, which refers to either C or T. In both cases the corresponding amino acid is Ile. Thus the invention includes a
15 polynucleotide encoding a CRCV S protein which comprises or consists of the sequence listed in Figure 3, and having C at position 3531. The invention also includes a polynucleotide encoding a CRCV S protein which comprises or consists of the sequence listed in Figure 3, and having T at position 3531.

- 20 The invention also includes a CRCV S polynucleotide comprising a fragment of the sequence listed in Figure 3 (SEQ ID NO: 3), that encodes a protein having at least one of the amino acids specific for CRCV S protein at the position listed in Table 1, or the complement thereof.

Preferably, the polynucleotide encoding the pol protein comprises or
25 consists of the sequence listed in Figure 1 (SEQ ID NO: 1), or the complement thereof.

The invention also includes a CRCV pol polynucleotide comprising a fragment of the sequence listed in Figure 1 (SEQ ID NO: 1) that encodes a protein having E at the position corresponding to position 4975 in the BCV genome, or the complement thereof.

- 5 Preferably, the polynucleotide encoding the HE protein comprises or consists of the sequence listed in Figure 13 (SEQ ID NO: 21), or the complement thereof.

The invention also includes a CRCV HE polynucleotide comprising a fragment of the sequence listed in Figure 13 (SEQ ID NO: 21) that encodes
10 a protein having one or more of the amino acid F at position 235, N at position 242, and L at position 253. The numbering of these three amino acid positions corresponds to that of BCV LY138 HE protein (Genbank Accession No. AF058942) in which residue number 1 is the initial M at the start of the BCV LY138 HE protein.

- 15 The polynucleotides as defined above are referred to herein as CRCV or CRCV-like polynucleotides of the invention.

A "CRCV-like polynucleotide" is a polynucleotide that does not have a base sequence identical to all or a fragment of the native CRCV cDNA sequence as listed in Figures 1, 3 and 13 (SEQ ID NOS: 1, 3 and 21), but
20 that encodes a CRCV or CRCV-like S pol or HE protein as defined above, or the complement thereof.

The CRCV is a positive strand RNA virus. The polynucleotide of the invention may be DNA or RNA. The RNA may be positive or negative strand RNA. The DNA may be single or double stranded DNA.

- 25 Suitable techniques for cloning and sequencing a cDNA from a positive strand RNA virus such as CRCV are well known in the art and are

described for example in Sambrook *et al* 2001, incorporated herein by reference.

The CRCV or CRCV-like polynucleotides of the invention may be any suitable size. However, for certain purposes, such as probing or amplifying,
5 it is preferred if the nucleic acid has fewer than 3,000, more preferably fewer than 1000, more preferably still from 10 to 100, and in further preference from 15 to 30 base pairs (if the nucleic acid is double-stranded) or bases (if the nucleic acid is single stranded). As is described more fully below, single-stranded DNA oligonucleotides, suitable for use as
10 hybridisation probes or as primers in a polymerase chain reaction, are particularly preferred.

Oligonucleotides that can specifically amplify, or hybridise to CRCV S, pol or HE polynucleotides, as opposed to BCV, HCV, HEV or enteric CCV S, pol or HE polynucleotides, are particularly preferred. Suitable
15 oligonucleotides can be determined by a person of skill in the art by reference to the nucleotide sequence comparisons in Figures 6, 8, 9 and 15.

It is appreciated that the CRCV or CRCV-like oligonucleotides may, even under highly stringent conditions, hybridise to nucleic acid, whether RNA or DNA, from HCV, BCV, and HEV as well as from CRCV. However, it is
20 preferred if the CRCV or CRCV-like oligonucleotides hybridise to nucleic acid from CRCV under more stringent conditions than to nucleic acid from HCV, BCV or HEV. This can either be determined experimentally or by a comparison of the oligonucleotide sequence with the respective CRCV, HCV, BCV and HEV sequences, as is well known to one of skill in the art
25 (Sambrook *et al* 2001).

It is also appreciated that the CRCV or CRCV-like oligonucleotides may hybridise to nucleic acid, whether RNA or DNA, from the enteric CCV as

well as from CRCV. However, it is preferred if the CRCV or CRCV-like oligonucleotides hybridise to nucleic acid from CRCV under more stringent conditions than to nucleic acid from enteric CCV. This can either be determined experimentally or by a comparison of the oligonucleotide
5 sequence with the respective sequences, as is well known to one of skill in the art (Sambrook *et al* 2001). Preferably, the oligonucleotides do not hybridise to nucleic acid from enteric CCV at all under stringent conditions (see below).

Conveniently, the CRCV or CRCV-like polynucleotides or oligonucleotides
10 further comprise a detectable label.

By "detectable label" is included any convenient radioactive label such as ^{32}P , ^{33}P or ^{35}S which can readily be incorporated into a nucleic acid molecule using well known methods; any convenient fluorescent or chemiluminescent label which can readily be incorporated into a nucleic
15 acid is also included. In addition the term "detectable label" also includes a moiety which can be detected by virtue of binding to another moiety (such as biotin which can be detected by binding to streptavidin); and a moiety, such as an enzyme, which can be detected by virtue of its ability to convert a colourless compound into a coloured compound, or *vice versa* (for
20 example, alkaline phosphatase can convert colourless *o*-nitrophenylphosphate into coloured *o*-nitrophenol). Conveniently, the nucleic acid probe may occupy a certain position in a fixed array and whether a nucleic acid hybridises to it can be determined by reference to the position of hybridisation in the fixed array.

25 Labelling with [^{32}P]dCTP may be carried out using a Rediprime[®] random primer labelling kit supplied by Amersham.

Primers which are suitable for use in a polymerase chain reaction (PCR; Saiki *et al* (1988) *Science* 239, 487-491) are preferred. Suitable PCR primers may have the following properties:

It is well known that the sequence at the 5' end of the oligonucleotide need
5 not match the target sequence to be amplified.

It is usual that the PCR primers do not contain any complementary structures with each other longer than 2 bases, especially at their 3' ends, as this feature may promote the formation of an artefactual product called "primer dimer". When the 3' ends of the two primers hybridise, they form a
10 "primed template" complex, and primer extension results in a short duplex product called "primer dimer".

Internal secondary structure should be avoided in primers. For symmetric PCR, a 40-60% G+C content is often recommended for both primers, with no long stretches of any one base. The classical melting temperature
15 calculations used in conjunction with DNA probe hybridization studies often predict that a given primer should anneal at a specific temperature or that the 72°C extension temperature will dissociate the primer/template hybrid prematurely. In practice, the hybrids are more effective in the PCR process than generally predicted by simple T_m calculations.

20 Optimum annealing temperatures may be determined empirically and may be higher than predicted. *Taq* DNA polymerase does have activity in the 37-55°C region, so primer extension will occur during the annealing step and the hybrid will be stabilised. The concentrations of the primers are equal in conventional (symmetric) PCR and, typically, within 0.1- to 1µM
25 range.

It will further be appreciated that if a control amplification reaction is to be carried out, for example using primers complementary to an ubiquitously expressed gene, that it may be beneficial for the products of the control and CRCV or CRCV-like products to be of different sizes, such that the two products may be distinguished by the detection means employed, for example by mobility on agarose gel electrophoresis. However, it may be desirable for the two products to be of similar size, for example both between 100 and 1000, or between 100 and 600 nucleotides long. This may aid simultaneous analysis of the products, for example by gel electrophoresis, and may also mean that the control and CRCV or CRCV-like amplification reactions may have similar performance characteristics, in terms, for example, of relative rates of accumulation of product at different stages during the reaction.

Any of the nucleic acid amplification protocols can be used in the method of the invention including the polymerase chain reaction, QB replicase and ligase chain reaction. Also, NASBA (nucleic acid sequence based amplification), also called 3SR, can be used as described in Compton (1991) *Nature* 350, 91-92 and *AIDS* (1993), Vol 7 (Suppl 2), S108 or SDA (strand displacement amplification) can be used as described in Walker *et al* (1992) *Nucl. Acids Res.* 20, 1691-1696. The polymerase chain reaction is particularly preferred because of its simplicity.

When a pair of suitable nucleic acids of the invention are used in a PCR it is convenient to detect the product by gel electrophoresis and ethidium bromide staining. As an alternative, it is convenient to use a labelled oligonucleotide capable of hybridising to the amplified DNA as a probe. When the amplification is by PCR the oligonucleotide probe hybridises to the interprimer sequence as defined by the two primers. The oligonucleotide probe is preferably between 10 and 50 nucleotides long,

more preferably between 15 and 30 nucleotides long. It may be longer than the amplified DNA or include one or both of the primers, but in this case, the hybridisation conditions should be such that the probe should not hybridise to the primers alone, but only to an amplified product that also
5 contains interprimer sequence that is capable of hybridising to the probe.

The probe may be labelled with a radionuclide such as ^{32}P , ^{33}P and ^{35}S using standard techniques, or may be labelled with a fluorescent dye. When the oligonucleotide probe is fluorescently labelled, the amplified DNA product may be detected in solution (see for example Balaguer *et al* (1991).
10 "Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescence adsorbent" *Anal. Biochem.* 195, 105-110 and Dilesare *et al* (1993) "A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantitation" *BioTechniques* 15, 152-157.

15 PCR products can also be detected using a probe which may have a fluorophore-quencher pair or may be attached to a solid support or may have a biotin tag or they may be detected using a combination of a capture probe and a detector probe.

Fluorophore-quencher pairs are particularly suited to quantitative
20 measurements of PCR reactions (eg RT-PCR). Fluorescence polarisation using a suitable probe may also be used to detect PCR products.

The invention also includes a vector comprising the CRCV or CRCV-like polynucleotide of the fourth aspect of the invention.

Typical prokaryotic vector plasmids are: pUC18, pUC19, pBR322 and
25 pBR329 available from Biorad Laboratories (Richmond, CA, USA); pTrc99A, pKK223-3, pKK233-3, pDR540 and pRIT5 available from

Pharmacia (Piscataway, NJ, USA); pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A available from Stratagene Cloning Systems (La Jolla, CA 92037, USA).

5 A typical mammalian cell vector plasmid is pSVL available from Pharmacia (Piscataway, NJ, USA). This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells. An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia (Piscataway, NJ, USA). This vector uses the glucocorticoid-inducible
10 promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems (La Jolla, CA 92037, USA). Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating
15 plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

Generally, the CRCV or CRCV-like polynucleotide of the invention is inserted into an expression vector, such as a plasmid, in proper orientation and
20 correct reading frame for expression. It may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host prior to insertion into the vector, although such controls are generally available in the expression vector. Thus, the polynucleotide of the invention insert may be operatively linked to an
25 appropriate promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters and the promoters of retroviral LTRs. Other suitable promoters will be known to the skilled artisan. The expression constructs desirably also

contain sites for transcription initiation and termination, and in the transcribed region, a ribosome binding site for translation (Hastings *et al*, International Patent No. WO 98/16643).

Methods well known to those skilled in the art can be used to construct
5 expression vectors containing the coding sequence and, for example
appropriate transcriptional or translational controls. One such method
involves ligation via homopolymer tails. Homopolymer polydA (or polydC)
tails are added to exposed 3' OH groups on the DNA fragment to be cloned by
terminal deoxynucleotidyl transferases. The fragment is then capable of
10 annealing to the polydT (or polydG) tails added to the ends of a linearised
plasmid vector. Gaps left following annealing can be filled by DNA
polymerase and the free ends joined by DNA ligase.

Another method involves ligation via cohesive ends. Compatible cohesive
ends can be generated on the DNA fragment and vector by the action of
15 suitable restriction enzymes. These ends will rapidly anneal through
complementary base pairing and remaining nicks can be closed by the action
of DNA ligase.

A further method uses synthetic molecules called linkers and adaptors. DNA
fragments with blunt ends are generated by bacteriophage T4 DNA
20 polymerase or *E.coli* DNA polymerase I which remove protruding 3' termini
and fill in recessed 3' ends. Synthetic linkers, pieces of blunt-ended double-
stranded DNA which contain recognition sequences for defined restriction
enzymes, can be ligated to blunt-ended DNA fragments by T4 DNA ligase.
They are subsequently digested with appropriate restriction enzymes to create
25 cohesive ends and ligated to an expression vector with compatible termini.
Adaptors are also chemically synthesised DNA fragments which contain one
blunt end used for ligation but which also possess one preformed cohesive
end.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the polynucleotide of the invention is to use the
5 polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression
10 vectors using methods known in the art.

The invention also includes a host cell transformed with the vector comprising the CRCV or CRCV-like polynucleotide. The host cell can be either prokaryotic or eukaryotic. If the CRCV or CRCV-like polynucleotide, in the vector, is to be expressed as a glycoprotein, the host
15 cell is a eukaryotic host cell, and preferably a mammalian host cell.

Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville,
20 MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred
25 mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1

cells available from the ATCC as CRL 1650. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a vector is accomplished by well known methods that typically depend on the type of vector used. With
5 regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110 and Sambrook *et al* (2001) *Molecular Cloning, A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory*
10 *Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* 275, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

15 Electroporation is also useful for transforming cells and is well known in the art for transforming yeast cell, bacterial cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is consistently
20 recovered following electroporation of the DNA-cell mixture suspended in 2.5x PEB using 6250V per cm at 25 μ FD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* 194, 182.

Physical methods may be used for introducing DNA into animal and plant
25 cells. For example, microinjection uses a very fine pipette to inject DNA molecules directly into the nucleus of the cells to be transformed. Another

example involves bombardment of the cells with high-velocity microprojectiles, usually particles of gold or tungsten that have been coated with DNA.

Successfully transformed cells, ie cells that contain a CRCV or CRCV-like DNA construct, can be identified by well known techniques. For example, one selection technique involves incorporating into the expression vector a DNA sequence (marker) that codes for a selectable trait in the transformed cell. These markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture, and tetracyclin, kanamycin or ampicillin resistance genes for culturing in *E.coli* and other bacteria. Alternatively, the gene for the selectable trait can be on another vector, which is used to co-transform the desired host cell.

The marker gene can be used to identify transformants but it is desirable to determine which of the cells contain recombinant DNA molecules and which contain self-ligated vector molecules. This can be achieved by using a cloning vector where insertion of a DNA fragment destroys the integrity of one of the genes present on the molecule. Recombinants can therefore be identified because of loss of function of that gene.

Another method of identifying successfully transformed cells involves growing the cells resulting from the introduction of an expression construct of the present invention to produce the CRCV or CRCV-like S, pol or HE protein. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression
5 vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal
10 (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

Host cells that have been transformed by the recombinant CRCV or CRCV-like polynucleotide, typically in a vector as described above, are then cultured for a sufficient time and under appropriate conditions known to those skilled
15 in the art in view of the teachings disclosed herein to permit the expression of the CRCV or CRCV-like protein encoded by the CRCV or CRCV-like polynucleotide, which can then be recovered.

The CRCV or CRCV-like protein can be recovered and purified from recombinant cell cultures by well-known methods including ammonium
20 sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

25 For example, for expression in a baculovirus system, recombinant DNA encoding the CRCV spike gene may be cloned into a suitable transfer vector such as pMelBac (Invitrogen). Co-transfection with baculovirus DNA (eg

Bac-N-Blue/Invitrogen) results in a recombinant baculovirus encoding the spike gene. Infection of a suitable insect cell line (e.g. Sf9, Sf21, High Five/Invitrogen) at an appropriate multiplicity of infection leads to expression of the recombinant spike protein. Protein expression is confirmed by western blotting or ELISA using appropriate reagents (e.g. convalescent canine serum or other virus specific antiserum).

The invention thus includes a method of obtaining a CRCV or CRCV-like protein encoded by the CRCV or CRCV-like polynucleotide of the present invention. The method comprises culturing the host cell comprising the CRCV or CRCV-like polynucleotide, typically in a vector; expressing the protein in the host cell, and purifying the protein. The invention further includes the protein obtainable by this method.

The invention thus also includes a method of obtaining a glycosylated CRCV or CRCV-like protein, typically an S protein, encoded by the CRCV or CRCV-like polynucleotide of the present invention. The method comprises culturing a eukaryotic, or more preferably mammalian, host cell comprising the CRCV or CRCV-like polynucleotide, typically in a vector; expressing the protein in the host cell; and purifying the glycosylated protein. The invention further includes the glycosylated protein obtainable by this method.

In a fifth aspect, the invention provides a method of making an anti-CRCV antibody comprising raising an immune response to a CRCV or CRCV-like S protein of the invention as described above in the first aspect of the invention in an animal, and preparing an antibody from the animal or from an immortal cell derived therefrom. Alternatively, the method may comprise selecting an antibody from an antibody-display library using a CRCV or CRCV-like S protein of the invention as described above in the first aspect of the invention.

Methods and techniques for producing a monoclonal antibody are well known to a person of skill in the art, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "*Monoclonal Hybridoma Antibodies: Techniques and*
5 *Applications*", J G R Hurrell (CRC Press, 1982), incorporated herein by reference.

Optionally, the method further comprises determining whether the antibody thus obtained has greater affinity for the CRCV S protein than for the BCV S protein, and preferably also whether the antibody has a greater affinity for
10 the CRCV S protein than for the HCV and HEV S proteins. Methods for determining the relative affinity of antibodies for antigens are known in the art.

The invention also includes an anti-CRCV antibody obtainable by the method of the fifth aspect of the invention, that has greater affinity for the
15 CRCV S protein than for the BCV S protein. Preferably, the antibody also has a greater affinity for the CRCV S protein than for the HCV and HEV S proteins.

The invention also includes a method of making an anti-CRCV antibody comprising raising an immune response to a CRCV or CRCV-like HE
20 protein of the invention as described above in the third aspect of the invention in an animal, and preparing an antibody from the animal or from an immortal cell derived therefrom. Alternatively, the method may comprise selecting an antibody from an antibody-display library using a CRCV or CRCV-like HE protein of the invention as described above in the
25 third aspect of the invention.

Optionally, the method further comprises determining whether the antibody thus obtained has greater affinity for the CRCV HE protein than for the

BCV HE protein, and preferably also whether the antibody has a greater affinity for the CRCV HE protein than for the HCV and HEV HE proteins. Methods for determining the relative affinity of antibodies for antigens are known in the art.

- 5 The invention also includes an anti-CRCV antibody obtainable by the method of the fifth aspect of the invention, that has greater affinity for the CRCV HE protein than for the BCV HE protein. Preferably, the antibody also has a greater affinity for the CRCV HE protein than for the HCV and HEV HE proteins.
- 10 Preferably, the antibody is a monoclonal antibody. However, the invention includes a monospecific anti-CRCV antibody. The antibody may be an antibody fragment, as described below.

The monoclonal or monospecific antibody may be a chimaeric antibody, as discussed by Neuberger *et al* (1988, *8th International Biotechnology Symposium* Part 2, 792-799).
15 The monoclonal or monospecific antibody may also be a "caninised" antibody, for example by inserting the CDR regions of mouse antibodies into the framework of canine antibodies.

The invention also includes anti-CRCV antibody fragments. The variable heavy (V_H) and variable light (V_L) domains of antibodies are involved in
20 antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies, in which variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984)
25 *Proc. Natl. Acad. Sci. USA* 81, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains.

- 5 These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs)
10 comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

- By "ScFv molecules" we mean molecules wherein the V_H and V_L partner
15 domains are linked via a flexible oligopeptide.

- The advantages of antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed.
20 Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the fragments.

- Whole antibodies, and F(ab')₂ fragments are "bivalent". By "bivalent" we mean that the antibodies and F(ab')₂ fragments have two antigen combining
25 sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

In a sixth aspect, the invention provides a method of determining whether a dog has been exposed to CRCV. The method comprises obtaining a suitable sample from the dog, and identifying CRCV or an anti-CRCV antibody in the sample. The method may be used as an aid in the diagnosis
5 of whether a dog has CIRD.

The invention includes a method of detecting, in a sample obtained from a dog, past exposure of the dog to CRCV, the method comprising obtaining a suitable sample from the dog, and identifying anti-CRCV antibodies in the sample.

10 In one preferred embodiment, the suitable sample can be any antibody containing sample such as serum, saliva, tracheal wash or bronchiolar lavage.

Preferably, the anti-CRCV antibody can be detected using a BCV, HCV, HEV or CRCV antigen, more preferably, using a BCV or CRCV antigen.

15 More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 75% identical with the amino acid sequence of the CRCV S protein (Figure 4 and SEQ ID NO: 4); an S protein whose amino acid sequence is at least 75% identical with the amino acid
20 sequence of the BCV S protein (Genbank Accession No. AF058942); HCV S protein (Genbank Accession No. L14643); to a coronavirus having an S protein at least 75% identical with BCV S protein (Genbank Accession No. AF058942), or a fragment thereof; or to a coronavirus having an S protein at least 75% identical with the CRCV S protein, or a fragment thereof.

25 More preferably, identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 75% identical with the amino

acid sequence of the BCV S protein, comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 80% identical, or at least 85% identical, or at least 90% identical, or at least 95% identical with the amino acid sequence of the BCV S protein (Genbank
5 Accession No. AF058942) or a fragment thereof.

More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to the BCV S protein (Genbank Accession No. AF058942).

Even more preferably, identifying an antibody that selectively binds to an S
10 protein whose amino acid sequence is at least 75% identical with the amino acid sequence of the CRCV S protein, comprises identifying an antibody that selectively binds to an S protein whose amino acid sequence is at least 80% identical, or at least 85% identical, or at least 90% identical, or at least 95% identical with the amino acid sequence of the CRCV S protein (Figure
15 4 and SEQ ID NO: 4) or a fragment thereof.

Yet more preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to a CRCV or CRCV-like S protein as defined in the first aspect of the invention.

Most preferably, identifying an anti-CRCV antibody in the sample
20 comprises identifying an antibody that selectively binds to the CRCV S protein as listed in Figure 4 (SEQ ID NO: 4), or a fragment thereof.

Similarly, identifying an anti-CRCV antibody in the sample may comprise identifying an antibody that selectively binds to an HE protein whose amino acid sequence is at least 90% identical with the partial amino acid sequence
25 of the CRCV HE protein (Figure 14 and SEQ ID NO: 22); to an HE protein whose amino acid sequence is at least 90% identical with the amino acid

sequence of the BCV HE protein (Genbank Accession No. AF058942) or the HECV HE protein (Genbank Accession No. L07747); to a coronavirus having an S protein at least 90% identical with BCV HE protein (Genbank Accession No. AF058942), or a fragment thereof; or to a coronavirus
5 having an HE protein at least 90% identical with the CRCV HE protein, or a fragment thereof.

More preferably, identifying an antibody that selectively binds to an HE protein whose amino acid sequence is at least 90% identical with the amino acid sequence of the BCV HE protein, comprises identifying an antibody
10 that selectively binds to an HE protein whose amino acid sequence is at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical with the amino acid sequence of the BCV HE protein (Genbank Accession No.
15 AF058942) or a fragment thereof.

More preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to the BCV HE protein (Genbank Accession No. AF058942).

Even more preferably, identifying an antibody that selectively binds to an
20 HE protein whose amino acid sequence is at least 90% identical with the partial amino acid sequence of the CRCV HE protein, comprises identifying an antibody that selectively binds to an HE protein whose partial amino acid sequence is at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least
25 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical with the partial amino acid sequence of the CRCV HE protein (Figure 13) or a fragment thereof.

Yet more preferably, identifying an anti-CRCV antibody in the sample comprises identifying an antibody that selectively binds to a CRCV or CRCV-like HE protein as defined in the third aspect of the invention.

Most preferably, identifying an anti-CRCV antibody in the sample
5 comprises identifying an antibody that selectively binds to the CRCV HE protein whose partial amino acid sequence is listed in Figure 14 (SEQ ID NO: 22), or a fragment thereof.

The invention includes a method of detecting CRCV in a sample obtained from a dog, the method comprising obtaining a suitable sample from the
10 dog, and identifying CRCV in the sample.

It is appreciated that there may be some naturally occurring sequence variation between different isolates of CRCV. The invention thus includes identifying CRCV isolates whose S, pol and HE genes and proteins have some sequence variation from the sequences provided in Figures 1 to 4 and
15 13 and 14. It is appreciated, however, that the same methods will be used to detect the variant isolates of CRCV, as well as the isolate characterised by the sequences listed in Figures 1 to 4 and 13 and 14.

In a preferred embodiment, the suitable sample can be a lung wash, tracheal wash, tonsillar swab or a biopsy or post-mortem sample from the respiratory
20 tract of the dog.

Preferably, in this embodiment, identifying CRCV comprises identifying a nucleic acid component of CRCV.

Typically, this will be performed by extracting RNA from the sample, and obtaining cDNA therefrom, for example as is described in Example 1.
25 Thereafter, a CRCV nucleic acid component is identified in the cDNA, for example using techniques involving high stringency hybridisation, specific

amplification, and nucleotide sequencing, as are well known to a person of skill in the art (Sambrook *et al* (2001) *supra*).

Preferably, identifying CRCV comprises identifying a polynucleotide that hybridises at high stringency to the BCV genome, such as the LY138 strain
5 genome (Genbank Accession No. AF058942) or a portion thereof.

Further preferably, identifying CRCV comprises identifying a polynucleotide that hybridises at high stringency to the CRCV S, pol or HE polynucleotides (Figures 1, 3 and 13) or a portion thereof.

By "hybridising at high stringency" is meant that the polynucleotide and the
10 nucleic acid to which it hybridises have sufficient nucleotide sequence similarity that they can hybridise under highly stringent conditions. As is well known in the art, the stringency of nucleic acid hybridisation depends on factors such as length of nucleic acid over which hybridisation occurs, degree of identity of the hybridising sequences and on factors such as
15 temperature, ionic strength and CG or AT content of the sequence.

Nucleic acids which can hybridise at high stringency to the CRCV cDNA molecule include nucleic acids which have >90% sequence identity, preferably those with >95% or >96% or >97% or >98, more preferably those with >99% sequence identity, over at least a portion of the CRCV
20 cDNA.

Typical highly stringent hybridisation conditions which lead to selective hybridisation are known in the art, for example those described in Sambrook *et al* 2001 (*supra*), incorporated herein by reference.

An example of a typical hybridisation solution when a nucleic acid is
25 immobilised on a nylon membrane and the probe nucleic acid is ≥ 500 bases is:

6 x SSC (saline sodium citrate)

0.5% sodium dodecyl sulphate (SDS)

100 µg/ml denatured, fragmented salmon sperm DNA

The hybridisation is performed at 68°C. The nylon membrane, with the
5 nucleic acid immobilised, may be washed at 68°C in 0.1 x SSC.

20 x SSC may be prepared in the following way. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H₂O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 litre with H₂O. Dispense into aliquots. Sterilise by autoclaving.

- 10 An example of a typical hybridisation solution when a nucleic acid is immobilised on a nylon membrane and the probe is an oligonucleotide of between 15 and 50 bases is:

3.0 M trimethylammonium chloride (TMACl)

0.01 M sodium phosphate (pH 6.8)

- 15 1 mM EDTA (pH 7.6)

0.5% SDS

100 µg/ml denatured, fragmented salmon sperm DNA

0.1% non-fat dried milk

- The optimal temperature for hybridisation is usually chosen to be 5°C
20 below the T_i for the given chain length. T_i is the irreversible melting temperature of the hybrid formed between the probe and its target sequence. Jacobs *et al* (1988) *Nucl. Acids Res.* 16, 4637 discusses the determination of T_is. The recommended hybridization temperature for 17-mers in 3M

TMACl is 48-50°C; for 19-mers, it is 55-57°C; and for 20-mers, it is 58-66°C.

Preferably, identifying CRCV comprises using a polynucleotide having at least 80%, or at least 85%, or at least 90%, or at least 95% identity with a
5 portion of the BCV genome (Genbank Accession No. AF058942).

More preferably, identifying CRCV comprises using a polynucleotide having at least 80%, or at least 85%, or at least 90%, or at least 95% identity with a portion of the CRCV S polynucleotide (Figure 3), or having at least 90%, or at least 95% identity with a portion of the CRCV pol
10 polynucleotide (Figure 1), or having at least 90%, or at least 95% identity with a portion of the CRCV HE polynucleotide (Figure 13).

More preferably, identifying CRCV comprises identifying a CRCV polynucleotide as defined above with respect to the fourth aspect of the invention.

15 Most preferably, identifying CRCV comprises identifying a CRCV polynucleotide comprising or consisting of a sequence listed in Figure 1 or Figure 3 or Figure 13, or a fragment thereof.

In another preferred embodiment, identifying CRCV comprises identifying a protein component of CRCV.

20 Preferably, identifying a protein component of CRCV comprises identifying a CRCV protein as defined above in the first or second or third aspects of the invention.

Most preferably, identifying a protein component of CRCV comprises identifying a CRCV protein comprising or consisting of the amino acid
25 sequence listed in Figure 2 or Figure 4 or Figure 14, or a fragment thereof.

Assaying a protein component of CRCV in a biological sample can occur using any art-known method. Preferred for assaying CRCV protein levels in a biological sample are antibody-based techniques.

Preferably, identifying a protein component of CRCV comprises using an antibody reactive with CRCV.

More preferably, the antibody reactive with CRCV is an anti-BCV antibody, an anti-HCV antibody, an anti-HEV antibody, or an anti-CRCV antibody obtainable or obtained by the methods of the fifth aspect of the invention.

For example, CRCV protein expression can be studied with classical immunohistological methods. In these, the specific recognition is provided by the primary antibody (polyclonal or monoclonal) but the secondary detection system can utilise fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunohistological staining of tissue section for pathological examination is obtained. Tissues can also be extracted, e.g., with urea and neutral detergent, for the liberation of CRCV protein for Western-blot or dot/slot assay (Jalkanen, M., *et al*, *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M., *et al*, *J. Cell. Biol.* 105:3087-3096 (1987)). In this technique, which is based on the use of cationic solid phases, quantitation of CRCV protein can be accomplished using isolated CRCV protein as a standard. This technique can also be applied to body fluid samples.

Other antibody-based methods useful for detecting CRCV protein expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, a CRCV reactive monoclonal antibody can be used both as an immunoabsorbent and as an enzyme-labeled probe to detect and quantify

the CRCV protein. The amount of CRCV protein present in the sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm. Such an ELISA for detecting a tumour antigen is described in Iacobelli *et al*, *Breast Cancer Research and Treatment* 11: 19-30 (1988). In another ELISA assay, two distinct specific monoclonal antibodies can be used to detect CRCV protein in a body fluid. In this assay, one of the antibodies is used as the immunoadsorbent and the other as the enzyme-labeled probe.

The above techniques may be conducted essentially as a "one-step" or "two-step" assay. The "one-step" assay involves contacting CRCV protein with immobilized antibody and, without washing, contacting the mixture with the labeled antibody. The "two-step" assay involves washing before contacting the mixture with the labeled antibody. Other conventional methods may also be employed as suitable. It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed from the sample.

Suitable enzyme labels include, for example, those from the oxidase group, which catalyze the production of hydrogen peroxide by reacting with substrate. Glucose oxidase is particularly preferred as it has good stability and its substrate (glucose) is readily available. Activity of an oxidase label may be assayed by measuring the concentration of hydrogen peroxide formed by the enzyme-labeled antibody/substrate reaction. Besides enzymes, other suitable labels include radioisotopes, such as iodine (^{125}I , ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium (^3H), indium (^{112}In), and technetium ($^{99\text{m}}\text{Tc}$), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

In a seventh aspect, the invention provides an immunosorbent assay for detecting anti-CRCV S or HE antibodies. The assay comprises a solid phase coated with a CRCV or CRCV-like S or HE protein, or coated with both CRCV or CRCV-like S and HE proteins as defined in the first and third aspects of the invention, or obtainable using the methods of the fourth aspect of the invention, or an antigenic fragment thereof, wherein anti-CRCV S or HE antibodies in a sample exposed to the solid phase will bind to the protein; and a detectable label conjugate which will bind to the anti-CRCV antibodies bound to the solid phase.

- 10 It is appreciated that an antigenic fragment of the CRCV or CRCV-like S protein that coats the solid phase is of sufficient size to be bound by an anti-CRCV S antibody, and which comprises at least one of the amino acids specific for CRCV S protein as listed in Table 1.

15 It is also appreciated that an antigenic fragment of the CRCV or CRCV-like HE protein that coats the solid phase is of sufficient size to be bound by an anti-CRCV HE antibody, and which comprises at least one of the three amino acids specific for CRCV HE protein as defined above.

Preferably, the CRCV or CRCV-like S or HE protein, or antigenic fragment thereof, that coats the solid phase is at least 10 amino acids in length. More preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, is at least 20, or at least 30, or at least 40, or at least 50, or at least 100, or at least 200, or at least 300, or at least 400 amino acids in length. The CRCV or CRCV-like S protein may be at least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1,000 amino acids in length.

Preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, that coats the solid phase is less than about 1200 amino acids in

length. More preferably, the CRCV or CRCV-like S protein, or antigenic fragment thereof, is less than about 1,100, or less than about 1,000, or less than about 900, or less than about 800, or less than about 700, or less than about 600, or less than about 500 amino acids in length. The CRCV or
5 CRCV-like S or HE protein may be less than about 400, or less than about 300, or less than about 200, or less than about 100, or less than about 50 amino acids in length.

Preferably, the solid phase is a microtitre well.

Further preferably, the conjugate comprises anti-dog antibody.

- 10 Preferably, the conjugate comprises an enzyme, for example horseradish peroxidase. Further preferably, the immunosorbent assay also comprises a substrate for the enzyme.

Further details of suitable immunosorbent assays and ELISAs are provided above.

- 15 The invention includes a kit of parts which include the components of the immunosorbent assay. The kit of parts may thus include a solid phase such as a microtitre plate, CRCV or CRCV-like S or HE protein or both for coating the solid phase, a detectable label conjugate, such as an anti-dog antibody, which will bind to anti-CRCV antibodies bound to the solid
20 phase. If the detectable label conjugate is an enzyme, the kit of parts may also include a substrate for the enzyme. The kit may also include a positive control sample that contains an anti-CRCV S or HE protein antibody, such as those described with reference to the fifth aspect of the invention, and a negative control sample.

- 25 The invention thus includes a solid substrate with a CRCV or CRCV-like S or HE protein as defined in the first and third aspects of the invention, or

obtainable using the methods of the fourth aspect of the invention, or an antigenic fragment thereof, attached thereto, for capturing anti-CRCV S or HE antibodies or both from a liquid sample, wherein anti-CRCV S or HE antibodies in a sample exposed to the solid substrate will bind to the S or
5 HE protein.

Typically, protein is coated on microtitre plates overnight at 4°C to 37°C, depending on the stability of the antigen. Unbound protein is washed off with a wash buffer such as phosphate buffered saline or Tris buffered saline. Serum or other samples are incubated on the plate, typically at 37°C for
10 between 1 and several hours. Unbound material is washed off, the plates are incubated with enzyme-labelled (e.g. horseradish peroxidase) antibody, such as anti-canine IgG or IgM for serum samples, or anti-canine IgA for lung washes, for 1 to several hours at 37°C. Unbound antibody is washed off and plates are incubated with a substrate such as OPD for about 10 min,
15 and the optical density measured in a photometer.

Preferably, the solid substrate is a microtitre well.

In an eighth aspect, the invention provides a vaccine composition for vaccinating dogs comprising (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus
20 having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV
25 protein or an immunogenic fragment thereof, or (vi) a coronavirus protein having at least 75% amino acid identity with a BCV protein or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof.

Preferably, the vaccine is packaged and presented for use in dogs.

When the vaccine comprises a coronavirus protein, or an immunogenic fragment thereof, the protein preferably has at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding
5 portion of a BCV or CRCV protein.

Preferably, the coronavirus protein is a BCV, HCV, HEV or CRCV protein, or a modification thereof.

Typical protein modifications include amino acid substitutions to improve the antigenicity of the vaccine. BCV, HCV and HEV proteins may be
10 modified to be more like a CRCV protein. For example, the spike protein of BCV, HCV or HEV may be modified to include a CRCV amino acid at any of differences shown in the comparison in Figure 10, or listed in Table 1. Additionally or alternatively, the HE protein of BCV, HCV or HEV may be modified to include a CRCV amino acid at any of the three CRCV-
15 specific residues as defined above.

Proteins in which one or more of the amino acid residues are chemically modified, may be used providing that the function of the protein, namely the production of specific antibodies *in vivo*, remains substantially unchanged. It is appreciated that synthesised proteins may be suitably modified before or
20 after their synthesised. Such modifications include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, and attaching amino acid protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the peptide from *in vivo* metabolism.

25 The protein may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently

antigenic themselves to obviate the use of a carrier. It may be advantageous for the protein to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow disulphide bonds to be formed. If the protein is
5 covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that the protein of the invention forms a loop.

According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. It is thought that the best carriers embody
10 (or, together with the antigen, create) a T-cell epitope. The peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the
15 nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys (SEQ ID NO: 52), beta-galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both. Alternatively, several copies of the same or different proteins of the invention may be cross-linked to one another;
20 in this situation there is no separate carrier as such, but a carrier function may be provided by such cross-linking. Suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal
25 cysteine residue (if present).

If the protein is prepared by expression of a suitable nucleotide sequence in a suitable host, then it may be advantageous to express it as a fusion product

with a peptide sequence which acts as a carrier. Kabigen's "Ecosec" system is an example of such an arrangement.

It is appreciated that the coronavirus component of the vaccine may be linked to other antigens to provide a dual effect.

- 5 Preferably, the coronavirus protein in the vaccine composition is an S protein. More preferably, the S protein is a CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV S protein, an HCV S protein, an HEV S protein, or an immunogenic fragment thereof.
- 10 Most preferably, the vaccine composition contains a CRCV S protein that comprises or consists of the amino acid sequence listed in Figure 4, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least at least 98%, or at least 99% amino acid sequence identity with the sequence listed in
- 15 Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

Additionally or alternatively, the vaccine composition may comprise

20 coronavirus proteins such as a hemagglutinin-esterase protein (HE) or an integral membrane protein (M), or the small membrane protein (E) (Lai MMC & Cavanagh D, (1997) "The molecular biology of coronaviruses" *Adv. Vir. Res.*, 48: 1-100).

In one embodiment, the HE, E or M proteins are BCV, HCV or HEV

25 proteins. In another embodiment, the HE, E or M proteins are CRCV proteins.

Preferably, the HE protein is a CRCV or CRCV-like HE protein as defined above in the third aspect of the invention or obtainable by the methods of the fourth aspect of the invention, or an immunogenic fragment thereof.

More preferably, the vaccine composition contains a CRCV HE protein that
5 comprises or consists of the partial amino acid sequence listed in Figure 14,
or an immunogenic fragment thereof having at least 97% identity with the
sequence listed in Figure 14. Preferably, the variant has at least at least
98%, or at least 99% amino acid sequence identity with the sequence listed
in Figure 14. More preferably the variant has at least 99.1%, or at least
10 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least
99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid
sequence identity with the sequence listed in Figure 14.

When the vaccine comprises a coronavirus, preferably the coronavirus
comprises an S protein with at least 80%, or at least 85%, or at least 90%, or
15 at least 95% amino acid identity with the BCV S protein. More preferably,
the coronavirus comprises an S protein with at least 80%, or at least 85%, or
at least 90%, or at least 95% amino acid identity with the CRCV S protein.

Additionally or alternatively, when the vaccine comprises a coronavirus,
preferably the coronavirus comprises an HE protein with at least 90% or at
20 least 95% amino acid identity with the BCV HE protein. More preferably,
the coronavirus comprises an HE protein with at least 96%, or at least 97%,
or at least 98%, or at least 99% amino acid identity with the CRCV HE
protein.

In another preferred embodiment, the vaccine composition comprises a
25 virus selected from BCV, HCV, HEV and CRCV, or a modification thereof.

It is appreciated that dog vaccines effective against a canine virus may be derived from a non-canine virus. For example US Patent No. 5,750,112 to Gill, and assigned to Solvay Animal Health Inc, discloses a vaccine against enteric canine coronavirus containing inactivated feline enteric coronavirus.

5 The disclosure of US 5,750,112 is incorporated herein by reference.

In one preferred embodiment, the virus is an inactivated virus. Methods for inactivating viruses for use in vaccines are well known in the art. Suitable methods include chemical methods, such as the use of beta proprio-lactone (BPL). Suitable inactivated bovine coronavirus vaccines may include
10 inactivated BCV which is a component of bovine vaccines such as "Rotovet Corona" from Schering-Plough (<http://www.ukvet.co.uk/rotovec/scour.htm>); "Lactovac" by Hoechst Roussel Vet Ltd, (Veterinary Formulary 5th Edition of the Veterinary Data Sheet Compendium); "First Defense" by Immuncell Corp, USA; "Scour
15 Bos 4" by Grand Laboraotries and "Scour Guard 3K" by Pfizer.

In an alternative embodiment, the virus is an attenuated virus. Methods for attenuating viruses for use in vaccines are well known in the art.

Preferably, the vaccine composition also comprises a pharmaceutically acceptable adjuvant.

20 Preferably, when the vaccine comprises a nucleic acid, the nucleic acid encoding the coronaviral protein or immunogenic fraction thereof, for use as a vaccine is a CRCV or CRCV-like S polynucleotide, or a CRCV or CRCV-like HE polynucleotide or both a CRCV or CRCV-like S and HE polynucleotide. More preferably, the nucleic acid comprises or consists of
25 the nucleotide sequence listed in Figure 3 or Figure 13, or fractions thereof.

For vaccine use, the CRCV or CRCV-like S or HE nucleic acid can be delivered in various replicating (e.g. recombinant adenovirus vaccine) or non-replicating (DNA vaccine) vectors.

In a preferred embodiment, the vaccine may contain recombinant CRCV or
5 CRCV-like S protein, as well as other immunogenic coronavirus proteins such as the HE protein.

As discussed above, several viral and bacterial agents are known to be associated with respiratory disease in dogs, including canine parainfluenza virus (CPIV), canine adenovirus type 2 (CAV-2), canine herpesvirus
10 (CHV), and *Bordetella bronchiseptica* (*B. bronchiseptica*).

In another preferred embodiment, the vaccine may contain recombinant CRCV or CRCV-like S or HE protein, as well as other pathogenic organisms involved in respiratory disease of dogs such as canine parainfluenzavirus, canine adenovirus type 2, the bacterium *Bordetella*
15 *bronchiseptica*, canine herpesvirus, human reovirus and mycoplasma species, or immunogenic proteins therefrom. Thus the vaccine may contain an agent capable of raising an immune response, such as the production of antibodies against CRCV, as well as against other pathogenic organisms involved in respiratory disease of dogs such as CPIV, CAV-2, *B.*
20 *bronchiseptica* and CHV.

In an embodiment, as well as containing an agent capable of stimulating the production of antibodies against CRCV, such as a CRCV or CRCV-like S or HE protein, the vaccine composition further comprises any one or more of:

- 25 (a) an agent capable of raising an immune response in a dog against CPIV;

(b) an agent capable of raising an immune response in a dog against CAV-2;

(c) an agent capable of raising an immune response in a dog against CHV; and

5 (d) an agent capable of raising an immune response in a dog against *B. bronchiseptica*.

Thus the vaccine composition can optionally also comprise any two, or any three or all four of these additional agents (a), (b), (c) and (d).

Typically, an agent capable of raising an immune response in a dog against
10 CPIV comprises inactivated or attenuated CPIV, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

Typically, an agent capable of raising an immune response in a dog against CAV-2 comprises inactivated or attenuated CAV-2, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

15 Canine adenovirus type 1 causes infectious hepatitis; canine adenovirus type 2 causes respiratory disease. It has been shown that CAV-1 provides cross-protection against CAV-2 and vice versa. The agent that raises an immune response in a dog against CAV-2 may therefore contain either CAV-1 or CAV-2, or an immunogenic fragment thereof. The vaccines
20 listed below contain CAV-2 except for EURICAN DHPPI, which does not specify the virus type used.

Suitable agents that raise an immune response in a dog against CPIV and CAV-2 are known to a person of skill in the art. For example, the following dog vaccines are licensed in the UK.

KAVAK DA₂PiP69 by Fort Dodge Animal Health is a live freeze dried vaccine containing attenuated strains of canine distemper virus, canine adenovirus type 2, canine parainfluenza type 2 and canine parvovirus grown in tissue culture.

- 5 KAVAK Parainfluenza by Fort Dodge Animal Health contains live freeze-dried vaccine derived from an attenuated strain of canine parainfluenza virus type 2 cultivated on an established homologous cell-line.

- NOBIVAC DHPPi by Intervet UK Limited is a live attenuated freeze-dried, virus vaccine containing canine distemper virus, canine adenovirus type 2,
10 canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

- NOBIVAC KC by Intervet UK Limited is a modified live freeze-dried vaccine containing *Bordetella bronchiseptica* strain B-C2 and canine parainfluenza virus strain Cornell (this is an intranasal vaccine).
15 Management authorisation number Vm 06376/4026.

EURICAN DHPPi by Merial Animal Health Ltd. is a combined live freeze-dried vaccine against canine distemper, infectious canine hepatitis, canine parvovirus and canine parainfluenza virus type 2.

- VANGUARD 7 by Pfizer Ltd. contains live attenuated canine distemper
20 virus (Snyder Hill strain), adenovirus (CAV-2 Manhattan strain), parainfluenza virus (NL-CPI-5 strain), canine parvovirus (NL-35-D) propagated in an established cell line, and an inactivated culture of *Leptospira canicola* and *Leptospira icterohaemorrhagiae*.

- QUANTUM DOG 7 by Schering-Plough Animal Health contains canine
25 distemper, adenovirus type 2, parvovirus, parainfluenza virus type 2 vaccine

(living) and inactivated *Leptospira canicola* and *Leptospira icterohaemorrhagiae* vaccine.

CANIGEN DHPPi by Virbac Ltd. is a live attenuated, freeze-dried, virus vaccine containing canine distemper virus, canine adenovirus (CAV2),
5 canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

CANIGEN Ppi by Virbac Ltd. is a live attenuated, freeze-dried virus vaccine containing canine parvovirus and canine parainfluenza virus grown in cell line tissue culture.

10 Typically, an agent capable of raising an immune response in a dog against CHV comprises inactivated or attenuated CHV, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

Suitable agents that raise an immune response in a dog against CHV are known to a person of skill in the art. For example, EURICAN Herpes 205
15 by Merial is a purified sub-unit vaccine against canine herpesvirus which is indicated for the active immunisation of pregnant bitches to prevent mortality, clinical signs and lesions in puppies resulting from canine herpesvirus infections acquired in the first days of life. It is not licensed for the vaccination of adult dogs for the prevention of respiratory disease.

20 Typically, an agent capable of raising an immune response in a dog against *B. bronchiseptica* comprises inactivated or attenuated *B. bronchiseptica*, or an immunogenic fragment thereof, or a nucleic acid encoding said immunogenic fraction.

Suitable agents that raise an immune response in a dog against *B.*
25 *bronchiseptica* are known to a person of skill in the art. For example, the following dog vaccines are licensed for use.

COUGHGUARD-B[®] by Pfizer Animal Health (U.S. Vet. Lic. No.: 189) contains an inactivated culture of *B. bronchiseptica*. It is for the immunisation of healthy dogs against disease caused by *B. bronchiseptica*, in particular kennel cough. COUGHGUARD-B[®] is prepared from a highly antigenic strain of *B. bronchiseptica* which has been inactivated and processed to be nontoxic when administered to dogs. The production method is reported to leave the immunogenic properties of *B. bronchiseptica* intact.

VANGUARD[®] 5/B by Pfizer Animal Health (U.S. Vet. Lic. No.: 189) contains attenuated strains of canine distemper virus (CDV), CAV-2, CPV, and canine parvovirus (CPV) propagated on an established canine cell line. The CPV antigen was attenuated by low passage on the canine cell line and at that passage level has immunogenic properties capable of overriding maternal antibodies. The vaccine is packaged in lyophilised form with inert gas in place of vacuum. The bacterin component containing inactivated whole cultures of *B. bronchiseptica* which is supplied as diluent. The *B. bronchiseptica* component in VANGUARD[®] 5/B is prepared from a highly antigenic strain which has been inactivated and processed to be nontoxic when administered to dogs.

NASAGUARD-B[™] by Pfizer Animal Health (U.S. Vet. Lic. No.: 112) is composed of an avirulent live culture of *B. bronchiseptica* bacteria.

PROGARD[®]-KC by Intervet is a modified live intranasal vaccine containing attenuated canine parainfluenza virus and *Bordetella bronchiseptica* avirulent live culture. PROGARD[®]-KC is presented in a desiccated form with sterile diluent provided for reconstitution. PROGARD[®]-KC is for vaccination of healthy, susceptible puppies and dogs for prevention of canine infectious tracheobronchitis ("kennel cough") due to canine parainfluenza virus and *B. bronchiseptica*.

- PROGARD®-KC PLUS by Intervet contains live culture of avirulent strains of *B. bronchiseptica*, attenuated canine adenovirus type 2 and parainfluenza virus for intranasal administration. Vaccination with PROGARD®-KC Plus stimulates rapid, local immunity in the respiratory tract, thereby inhibiting infection at the port of entry as well as preventing clinical signs. In addition to local immunity, it also stimulates systemic immunity within three weeks of intranasal administration. The small volume (0.4 ml) and one nostril application of PROGARD®-KC Plus provide for ease in vaccination, particularly in small breeds and young puppies. PROGARD®-KC Plus is presented in a desiccated form with sterile diluent provided for reconstitution. PROGARD®-KC Plus is for vaccination of healthy dogs and puppies three weeks of age or older for prevention of canine infectious tracheobronchitis ("kennel cough") due to canine adenovirus type 2, parainfluenza virus and *B. bronchiseptica*.
- Intrac by Intervet is a freeze dried modified live vaccine, containing *B. bronchiseptica* strain S 55, for intranasal administration. Product licence number PL 0201/4011

Nobivac KC, described above, also contains *B. bronchiseptica*.

- Vaccination would be useful especially but not exclusively for dogs prior to entry into a boarding kennel or for the vaccination of dogs in breeding facilities.

A typical dose of a vaccine comprised of recombinant protein is about 5-10 µg. A typical dose of a vaccine comprised of inactivated virus is about 1-10 mg.

- In a ninth aspect, the invention provides the use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or

(ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for stimulating an immune response against CRCV in a dog.

The invention includes the use of (i) a coronavirus having an S protein with at least 75% amino acid identity with CRCV S protein, or (ii) a coronavirus having an S protein with at least 75% amino acid identity with BCV S protein, or (iii) a coronavirus having an HE protein with at least 90% amino acid identity with CRCV HE protein, or (iv) a coronavirus having an HE protein with at least 90% amino acid identity with BCV HE protein, or (v) a coronavirus protein having at least 75% amino acid identity with a CRCV protein or an immunogenic fragment thereof, or (vi) a coronaviral protein having at least 75% amino acid identity with a BCV protein, or an immunogenic fragment thereof, or (vii) a nucleic acid encoding said coronaviral protein or immunogenic fraction thereof, in the preparation of a medicament for prophylaxis of respiratory disease in a dog, typically CIRDC.

When a coronavirus protein, or an immunogenic fragment thereof, is used in the preparation of the medicament, the protein preferably has at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding portion of a BCV protein. Preferably the protein has

at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the corresponding portion of a CRCV protein.

Preferably, the coronaviral protein used in the preparation of the medicament is a BCV, HCV, HEV or CRCV protein, or a modification thereof, as described above with reference to the eighth aspect of the invention.

More preferably, the coronaviral protein used in the preparation of the medicament is an S protein. Yet more preferably, the S protein comprises an CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV S protein, an HCV S protein, or an immunogenic fragment thereof.

Most preferably, the coronaviral protein used in the preparation of the medicament comprises or consists of the amino acid sequence listed in Figure 4, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 4. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence listed in Figure 4. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the sequence listed in Figure 4.

Additionally or alternatively, the coronaviral protein used in the preparation of the medicament may comprise HE, E, M or N coronavirus proteins. In one embodiment, the HE, E, M or N proteins are BCV, HCV or HEV proteins. In another embodiment, the HE, E, M or N proteins are CRCV proteins.

Typically, the HE protein comprises an CRCV or CRCV-like HE protein as defined above in the third aspect of the invention or obtainable by the methods of the fourth aspect of the invention, a BCV HE protein, an HCV HE protein, or an immunogenic fragment thereof.

- 5 Preferably, the coronaviral HE protein used in the preparation of the medicament comprises or consists of the partial amino acid sequence listed in Figure 14, or an immunogenic fragment thereof having at least 97% identity with the sequence listed in Figure 14. Preferably, the variant has at least 98%, or at least 99% amino acid sequence identity with the sequence
10 listed in Figure 14. More preferably the variant has at least 99.1%, or at least 99.2%, or at least 99.3%, or at least 99.4%, or at least 99.5%, or at least 99.6%, or at least 99.7%, or at least 99.8%, or at least 99.9% amino acid sequence identity with the partial sequence listed in Figure 14.

- When a coronavirus is used in the preparation of the medicament, the
15 coronavirus preferably comprises an S protein with at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the BCV S protein. More preferably the coronavirus comprises an S protein with at least 80%, or at least 85%, or at least 90%, or at least 95% amino acid identity with the CRCV S protein.

- 20 Additionally or alternatively, the coronavirus may comprise an HE protein with at least 90%, or at least 95% amino acid identity with the BCV HE protein. More preferably the coronavirus comprises an HE protein with at least 96%, or at least 97%, or at least 98%, or at least 99% amino acid identity with the CRCV HE protein.

- 25 In a tenth aspect, the invention provides a CRCV or CRCV-like S protein as defined above in the first aspect of the invention or obtainable by the

methods of the fourth aspect of the invention, for use in medicine.
Typically, the S protein will be used in veterinary medicine.

The invention includes a CRCV or CRCV-like HE protein as defined above
in the third aspect of the invention or obtainable by the methods of the
5 fourth aspect of the invention, for use in medicine. Typically, the HE
protein will be used in veterinary medicine.

In an eleventh aspect, the invention provides a method of vaccinating a dog
against CRCV, the method comprising administering to the dog a vaccine
composition as described above in the ninth aspect of the invention.

10 Typically, the vaccine will be administered *via* the intramuscular,
subcutaneous or intranasal routes

In another embodiment, a dog can passively acquire immunity against
CRCV by being administered an antibody that reacts with CRCV. The
antibody that reacts with CRCV may be an anti-BCV, anti-HCV antibody,
15 but is preferably an anti-CRCV antibody. Preferably, the antibody that
reacts with CRCV is an anti-S protein antibody an anti-HE protein antibody.
Most preferably, the antibody that reacts with CRCV is an anti-CRCV S or
HE protein antibody as described in the fifth aspect of the invention.

In a twelfth aspect, the invention provides a method for combating the
20 spread of CRCV between dogs comprising determining whether a dog is
infected with CRCV according to the methods as described above in the
sixth aspect of the invention, or using the immunosorbent assay or solid
substrate as described above in the seventh aspect of the invention, and, if
the dog is infected with CRCV, quarantining the dog.

25 By "quarantining" a dog we include the meaning of keeping the dog
separate from all other dogs. We also include the meaning of keeping the

dog separate from dogs that have not been vaccinated against CRCV, which can be performed as described above. We also include the meaning of keeping the dog separate from dogs that have not been infected by CRCV, which can be determined as described above.

- 5 In a thirteenth aspect, the invention provides a method for combating the spread of CRCV between dogs comprising determining whether a dog is infected with CRCV according to the methods described above in the sixth aspect of the invention, or using the immunosorbent assay or solid substrate as described above in the seventh aspect of the invention, and, if the dog is
10 infected with CRCV, vaccinating other dogs that have been, are, or are likely to be in contact with the dog.

- A fourteenth aspect of the invention provides a method for identifying a test vaccine capable of preventing or reducing the incidence of canine infectious respiratory disease (CIRD) in dogs. The method comprises (a) determining
15 whether a dog has been exposed to CRCV, typically according to the methods described above in the sixth aspect of the invention or using the immunosorbent assay or solid substrate as described above in the seventh aspect of the invention, (b) if the dog has not been exposed to CRCV, administering the test vaccine to the dog, (c) inoculating the dog with
20 CRCV, and (d) determining whether the dog develops CIRD. The absence of CIRD in step (d) indicates that the test vaccine is capable of preventing CIRD.

Typically, this method is performed on a set of dogs.

- Preferably, the method involves the use of a set of control dog which are not
25 administered the test vaccine in step (b). The significantly lower incidence of CIRD in the set of dogs that has been administered the test vaccine than

in the control set indicates that the test vaccine is capable of preventing or reducing the incidence of CIRDC.

The invention also includes a vaccine identified by this method.

All of the documents referred to herein are incorporated herein, in their entirety, by reference.

The invention will now be described in more detail with the aid of the following Figures and Examples.

Figure 1

Partial nucleotide sequence (250 residues) of the CRCV polymerase (pol) cDNA (SEQ ID NO: 1).

Figure 2

Partial amino acid sequence (83 residues) of the CRCV pol protein (SEQ ID NO: 2), derived from the nucleotide sequence of Figure 1.

Figure 3

Nucleotide sequence (4092 residues) of the CRCV Spike (S) cDNA (SEQ ID NO: 3). The Y at position 3531 refers to either C or T.

Figure 4

Amino acid sequence (1363 residues) of the CRCV S protein (SEQ ID NO: 4), derived from the nucleotide sequence of Figure 3.

Figure 5

Consensus tree for cDNA sequences from a 250 nucleotide region of the polymerase gene of 12 coronaviruses. The sequence obtained from the canine respiratory coronavirus is designated T101. The numbers indicate
5 bootstrap values obtained by analysis of 100 data sets.

BCV: bovine coronavirus, CCV: canine coronavirus, FIPV: feline infectious peritonitis virus, HEV: hemagglutinating encephalomyelitis virus, IBV: infectious bronchitis virus, MHV: mouse hepatitis virus, OC43: human coronavirus strain OC43, SDAV: sialodacryoadenitis virus, TCV: turkey
10 coronavirus, TGEV: transmissible gastroenteritis virus, 229E: human coronavirus strain 229E, T101: canine respiratory coronavirus (PCR product from tracheal sample T101)

Figure 6

CLUSTAL X (1.8) multiple sequence alignment of the 250 nucleotide
15 partial sequence of the pol cDNA of CRCV (sample T101, SEQ ID NO: 1), BCV (SEQ ID NO: 5), HCV strain OC43 (SEQ ID NO: 6), HEV (SEQ ID NO: 7) and CCV (enteric CCV, SEQ ID NO: 8).

Figure 7

CLUSTAL X (1.8) multiple sequence alignment of the 83 amino acid
20 partial sequence of the pol protein of CRCV (protCRCVpol, SEQ ID NO: 2) with HCV (protHCVpoly, SEQ ID NO: 9), HEV (protHEVpoly, SEQ ID NO: 10), BCV (protBCVpoly, SEQ ID NO: 11) and CECV (enteric CCV, protCECVpol, SEQ ID NO: 12).

Figure 8

CLUSTAL X (1.8) sequence alignment of the nucleotide sequence of the CRCV spike cDNA (CRCVspike, SEQ ID NO: 3) and enteric CCV spike cDNA (CECVspike, SEQ ID NO: 13).

5 **Figure 9**

CLUSTAL X (1.8) multiple sequence alignment of the 4092 nucleotides of the CRCV spike cDNA (CRCVspike, SEQ ID NO: 3) sequence with BCV (BCVspike, SEQ ID NO: 14), HCV (HCVspike, SEQ ID NO: 15) and HEV (HEVspike, SEQ ID NO: 16) spike cDNAs. The Y at position 3531 in the
10 CRCV sequence refers to either C or T.

Figure 10

CLUSTAL X (1.8) multiple sequence alignment of the 1363 amino acid sequence of the CRCV spike protein (CRCVspikepr, SEQ ID NO: 4) with BCV (BCVspikepro, SEQ ID NO: 17), HCV (HCVspikepro, SEQ ID NO: 18), HEV (HEVspikepro, SEQ ID NO: 19) and enteric CCV (CECVspikepr, SEQ ID NO: 20) spike proteins.

Figure 11

RT-PCR using nested set of primers (Spike 1 and 2 (SEQ ID NOS: 34 and 35) followed by Spike 3 and 4 (SEQ ID NOS: 36 and 37)). BCV: Bovine coronavirus positive control sample; A72: Coronavirus negative A72 cells;
20 H₂O: PCR mix without DNA; T5 – T21: Tracheal samples of study dogs. The agarose gel electrophoresis shows PCR products of the expected size of 442bp for the positive control (BCV) and samples T12 and T21.

Figure 12

Comparison of the prevalence of respiratory disease in two groups of dogs. Dogs in group 1 were positive for serum antibodies to respiratory coronavirus on day of entry into the kennel, dogs in group 2 were negative.

5 The graph shows the percentage of dogs developing respiratory disease in group 1 compared to group2 ($p < 0.001$). n is the total number of dogs in each group.

Figure 13

Partial nucleotide sequence (497 residues) of the CRCV hemagglutinin/esterase (HE) gene (SEQ ID NO: 21). The sequence

10 corresponds to nucleotides 418 to 914 of the HE genes of BCV (GenBank M84486) and HCV OC43 (GenBank Accession No. M76373).

Figure 14

Partial amino acid sequence (165 residues) of the CRCV HE protein (SEQ

15 ID NO: 22), derived from the nucleotide sequence of Figure 13. This sequence corresponds to amino acid residues 140 to 304 of BCV (GenBank M84486) and HCV OC43 (GenBank Accession No. M76373).

Figure 15

CLUSTAL X (1.8) multiple sequence alignment of a 497 nucleotide partial

20 sequence of the hemagglutinin/esterase (HE) gene of CRCV (canine respiratory coronavirus, SEQ ID NO: 21) with BCV (bovine coronavirus strain LY138, (SEQ ID NO: 23, taken from Genbank Accession No. AF058942), OC43 (human coronavirus strain OC43, SEQ ID NO: 24 taken from Genbank Accession No. M76373), HECV (human enteric coronavirus,

25 SEQ ID NO: 25, taken from Genbank Accession No. L07747), and HEV

(hemagglutinating encephalomyelitis virus, SEQ ID NO: 26, taken from Genbank Accession No. AF481863).

Figure 16

CLUSTAL X (1.8) multiple sequence alignment of a 165 amino acid partial
5 sequence of the HE protein of CRCV (canine respiratory coronavirus, (SEQ
ID NO: 22) with BCV (bovine coronavirus strain LY138, SEQ ID NO: 27,
taken from Genbank Accession No. AF058942), OC43 (human coronavirus
strain OC43, SEQ ID NO: 28, taken from Genbank Accession No.
M76373), HECV (human enteric coronavirus, SEQ ID NO: 29, taken from
10 Genbank Accession No. L07747), and HEV (hemagglutinating
encephalomyelitis virus, SEQ ID NO: 30, taken from Genbank Accession
No. AF481863). The three CRCV-specific amino acids F, N and L are
indicated in bold and are underlined.

Figure 17

15 RT-PCR using consensus primers HE1 (SEQ ID NO: 38) and HE2 (SEQ ID
NO: 39) directed to the HE gene of BCV and HCV (strain OC43). The
agarose gel electrophoresis shows a PCR product of the expected size of
497bp for the BCV positive control and for four tracheal samples from
study dogs (T90, T91, T101 and T105), and not for coronavirus-negative
20 A72 cells or the PCR mix without DNA (H₂O). 1 kb indicates a molecular
size standard (Promega).

Figure 18

CRCV Spike gene cloning strategy.

Example 1: Detection of a novel coronavirus associated with canine infectious respiratory disease

Summary

An investigation into the causes of canine infectious respiratory disease
5 (CIRD) was carried out in a large re-homing kennel. Tissue samples taken
from the respiratory tract of diseased dogs were tested for the presence of
coronaviruses using RT-PCR with conserved primers for the polymerase
gene. Sequence analysis of four positive samples showed the presence of a
10 novel coronavirus with high similarity to both bovine and human
coronavirus (strain OC43) in their polymerase and spike genes whereas
there was a low similarity to comparable genes in the enteric canine
coronavirus. This canine respiratory coronavirus (CRCV) was detected by
RT-PCR in 32/119 tracheal and 20/119 lung samples with the highest
prevalence being detected in dogs with mild clinical symptoms. Serological
15 analysis showed that the presence of antibodies against CRCV on the day of
entry into the kennel decreased the risk of developing respiratory disease.

Materials and Methods

Study population

Dogs from a well-established re-homing kennel with a history of endemic
20 respiratory disease were monitored for this study. On entry into the kennel,
all dogs were vaccinated with KAVAK DA₂ PiP69 (Fort Dodge) a live
attenuated vaccine for distemper virus, canine adenovirus type 2, canine
parainfluenzavirus and canine parvovirus. Also, a killed leptospirosis
vaccine was used (Fort Dodge). The health status of each dog was assessed
25 twice a day by a veterinary clinician and the respiratory symptoms were
graded as follows: 1: no respiratory signs, 2: mild cough, 3: cough and nasal

discharge, 4: cough, nasal discharge and inappetence, 5: bronchopneumonia. The overall health status of the dogs was graded as follows: 1: good health, 2: poor health, 3: very poor health. The age, breed and sex of the dogs were recorded.

- 5 For 119 dogs a full post mortem examination was performed. The tissue samples were stored at -70°C until further use.

Serum samples were collected from 111 dogs on day of entry into the rehoming kennel. For 81 dogs a follow-up serum was available on day 7 and for 111 dogs a serum was available on day 21 after entry.

- 10 Of the 111 dogs, 30 remained healthy during the 21 days between the first and the last serum sample whereas 81 dogs developed respiratory disease.

- Sera from 35 dogs housed elsewhere were obtained from the diagnostic service of the Royal Veterinary College. These sera had been submitted for biochemical analysis for various reasons. Five of these sera were from 18-month-old beagles with no history of respiratory disease. Sera were routinely stored at -20°C .
- 15

RNA extraction and RT-PCR

- RNA was extracted from tracheal and lung tissue of 119 dogs using TriReagent (Sigma). Approximately 25-50 mg of homogenised tissue was used and RNA was extracted as recommended by the manufacturer.
- 20

Synthesis of cDNA was performed using Random Hexamers (Roche) and ImPromII reverse transcriptase (Promega).

The polymerase gene of coronaviruses is known to be highly conserved, and has previously been used for phylogenetic analysis of this virus family

(Stephensen *et al.*, 1999). For the detection of coronaviruses a modification of the primers 2Bp and 4Bm directed against the polymerase gene as described by Stephensen *et al.* (1999) were used (Conscoro5: 5' -ACT-CAR-ATG-AAT-TTG-AAA-TAT-GC (SEQ ID NO: 31); and Conscoro6: 5' -TCA-CAC-TTA-GGA-TAR-TCC-CA (SEQ ID NO: 32)).

PCR was performed using Taq polymerase (Promega) in the provided reaction buffer containing a final concentration of 2.5 mM MgCl₂ and 0.5µM of primers. For PCR with the primers Conscoro5 and Conscoro6 the following temperature profile was used: After denaturation at 95°C for 5 min, 10 cycles were carried out at 95°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 1min. This was followed by 10 cycles using an annealing temperature of 45°C, 10 cycles at an annealing temperature of 50°C and 10 cycles at an annealing temperature of 53°C followed by a final extension at 72°C for 10 min.

A 20µl fraction of the PCR product was analysed on a 1.5% agarose gel and blotted onto a nylon membrane (Roche) after electrophoresis. The nylon membrane was hybridised with an oligonucleotide probe specific for the PCR product at 37°C overnight (Probe Conscoro: AAG-TTT-TAT-GGY-GGY-TGG-GA (SEQ ID NO: 33)). The probe was 3'A-tailed with Digoxigenin-dUTP and was detected using anti-Digoxigenin conjugate and CSPD chemoluminescent substrate (Roche).

Primer sequences specific for the spike gene were derived from an alignment of the spike region of bovine coronavirus strain LY-138 (AF058942) and human coronavirus strain OC43 (L14643).

A PCR was performed with the primers Spike 1 and Spike 2, followed by a nested PCR using the primers Spike 3 and Spike 4 and 2µl of the product of the first amplification.

The numbers in brackets refer to the nucleotide position in the bovine coronavirus genome.

Spike 1: 5'-CTT-ATA-AGT-GCC-CCC-AAA-CTA-AAT (25291-25314)

Spike 2: 5' -CCT-ACT-GTG-AGA-TCA-CAT-GTT-TG (25912-25890)

5 Spike 3: 5' -GTT-GGC-ATA-GGT-GAG-CAC-CTG (25320-25339)

Spike 4: 5' -GCA-ATG-CTG-GTT-CGG-AAG-AG (25762-25742)

Oligonucleotide Spike 1 has SEQ ID NO: 34, Spike 2 has SEQ ID NO: 35, Spike 3 has SEQ ID NO: 36, Spike 4 has SEQ ID NO: 37.

10 The temperature profile used was denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 40 sec and elongation at 72°C for 1 min. The final extension was performed at 72°C for 10 min. The nested PCR produced a 442bp fragment.

15 PCR products were cloned into the pGEM-T-easy vector (Promega) and sequenced using the Thermo sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia) using Cy5 labelled primers.

Phylogenetic analysis

20 An alignment of the 250 bp cDNA sequence from the polymerase gene to the corresponding sequences of 11 coronaviruses was performed using ClustalX (Thompson *et al.*, 1997).

The phylogenetic relationship to known coronaviruses was analysed using the Phylip 3.6 package (Felsenstein, 1989). The alignments were followed by a bootstrap analysis using the Seqboot programme. The obtained data sets were used for a maximum parsimony analysis using the DNAPars

programme and a consensus tree was calculated using Consense. The resulting trees were drawn using the Treeview programme (Page, 1996).

ELISA

ELISA antigen for bovine coronavirus or enteric canine coronavirus (CECV) (the antigens are a preparation from virus infected cell cultures obtained from Churchill Applied Biosciences, Huntingdon, UK) was resuspended in PBS at the concentration recommended by the manufacturer and incubated on 96 well plates (Falcon) overnight at 37°C.

The plates were washed with PBS and blocked with PBS containing 5% skimmed milk powder for 30 min. The sera were diluted 1:100 in blocking buffer and incubated on the plates for 1h. After washing with PBS/ 0.05% Tween 20 (Sigma), a peroxidase labelled rabbit anti-dog IgG conjugate (Sigma) was added (1:5000 in PBS/0.05% Tween 20) for 1 h. The plates were incubated with colour substrate (OPD, Sigma) for 10 min and the reaction was stopped by adding 2M H₂SO₄. The adsorption was determined in an ELISA photometer at 492nm.

Virus culture

Virus isolation is performed on canine adult lung fibroblasts (passage 3 to 7), MDCK and A72 cells. (It is appreciated, however, that virus isolation could be performed using primary cells or cell lines such as MDCK or A72 (canine), MDBK (bovine), HRT-18 (human rectal tumour cell line) and Vero (African Green Monkey). The lung fibroblasts are maintained in MEM with 20% fetal calf serum (FCS), MDCK and A72 cells are maintained in MEM with 5% FCS. Tracheal tissue samples (approx. 25mg) are homogenised using a scalpel and mixed vigorously in 1ml MEM containing Penicillin (100U/ml), Streptomycin (0.1mg/ml), Amphotericin B

(2.5µg/ml) and Trypsin (1µg/ml). The samples are centrifuged at 13000 rpm for 10 min. and the supernatant is used to inoculate cell cultures. After 30 min. at 37°C the supernatant is removed and maintenance medium added to the cultures. The cultures are passaged three times in the absence of a
5 cytopathic effect. Then, RNA is extracted from the cells and RT-PCR to detect the presence of CRCV is performed.

Statistical analysis

The data were analysed using the chi-square test or Fisher's exact test and p values below 0.05 were considered statistically significant.

10 Results

PCR using consensus primers for the coronavirus RNA polymerase gene

Using the primers Conscore5 and Conscore6, cDNA obtained from 40 tracheal samples was analysed by RT-PCR.

15 Out of these, seven were found to be positive by PCR and subsequent hybridisation (17.5%).

The PCR products were cloned and sequenced (Figures 1 and 2) and the sequence data were compared to available viral sequences using the FASTA search program (Pearson, 1990).

20 Comparison of the coronavirus cDNA polymerase sequence obtained from four of the canine tracheal samples to other coronavirus sequences revealed that they were most similar to sequence data from BCV strain Quebec and LY138 (Genbank Accession Nos. AF220295 and AF058942, respectively) and human coronavirus strain OC43 (Genbank Accession No. AF124989).

The similarity in the analysed 250 bp sequence was 98.8% for BCV Quebec, and 98.4% for BCV LY138 and the HCV pol genes, whereas it was only 68.53% for CCV strain 1-71 pol gene (Figures 6 and 7).

5 An alignment of the novel sequence with the corresponding sequences of 11 coronaviruses and phylogenetic analysis using the maximum parsimony method resulted in the consensus tree shown in Figure 5. The cDNA sequence obtained from a tracheal sample (T101) was found on a common branch with bovine coronavirus, human coronavirus-OC43 and hemagglutinating encephalomyelitis virus.

10 The virus was called canine respiratory coronavirus (CRCV).

PCR using primers for the spike gene

For further analysis of the RNA sequence of CRCV, an alignment of the RNA for the spike gene of the bovine coronavirus LY 138 strain (AF058942) and the human coronavirus OC43 strain (L14643) was
15 performed using Clustal X (Thompson *et al.*, 1997). Consensus regions were chosen for the selection of the nested primer sets Spike 1-2 and Spike 3-4 (Figure 11). PCR analysis was performed with the cDNA obtained from 119 tracheal and lung samples using these nested primers.

In total 32 tracheal samples (26.9%) and 20 lung samples (16.8%) were
20 found positive by nested PCR. For eight dogs a positive PCR result was obtained for both, trachea and lung.

Sequence analysis of the PCR products obtained from tissues of six different dogs showed identical DNA sequences for these cDNAs (Figures 3 and 4). A comparison to known coronavirus spike sequences using the
25 FASTA program revealed a 98.1% similarity to bovine coronavirus and a 97.8% similarity to human coronavirus OC43 (Figures 9 and 10).

PCR using primers for the HE gene

Bovine coronavirus and other group II coronaviruses contain an additional structural protein, the hemagglutinin/esterase (HE). Because of the high similarity of CRCV with BCV, we analysed the presence of an HE gene in
5 CRCV.

An alignment of the HE genes sequences of BCV and HCV OC43 was used to design the primers HE1 and HE2 (Table 2). Four tracheal samples that had previously been identified as positive for coronavirus RNA by RT-PCR with primers for the S gene were tested by RT-PCR with the primer set for
10 the HE gene. All four samples showed a PCR band of the expected size after agarose gel electrophoresis (Figure 17).

Table 2: Primers designed from an alignment of the hemagglutinin/esterase genes of BCV (GenBank Accession No. M84486) and HCV OC43 (GenBank Accession No. M76373)

15

Name	Sequence	Location in BCV HE gene
HE 1	5'-TAT-CGC-AGC-CTT-ACT-TTT-GT	418-437
HE 2	5'-ACC-GCC-GTC-ATG-TTA-TCA-G	914-896

Primer HE1 has SEQ ID No: 38 and HE2 has SEQ ID No: 39. The sequence of the CRCV PCR product obtained using primers HE 1 and HE 2 is given in Figure 13 (SEQ ID No: 21), and its predicted amino acid
20 sequence is listed in Figure 14 (SEQ ID No: 22). A comparison of these nucleotide and amino acid sequences with the corresponding fragments of other related coronaviruses is shown in Figures 15 and 16. Three amino acids were shown to be unique to CRCV, as shown in Table 3.

Table 3: Unique amino acids in CRCV HE gene

Amino acid in CRCV	Amino acid in BCV/ HECV/ HCV/ HEV	Position in BCV/ HECV/ HCV/ HEV	Position in PCR product HE1-HE2
F (Phe)	L (Leu)	235	96
N (Asn)	T (Thr)	242	103
L (Leu)	V (Val)	253	114

The amino acid positions in BCV, HECV, HCV and HEV are numbered from the initial M (which is number 1) at the start of the BCV and HCV OC43 HE proteins (GenBank Accession Nos. M84486 and M76373, respectively).

5 Association of PCR positive samples with respiratory signs

Using primers for the spike gene, tracheal and lung samples from 119 dogs were analysed by RT-PCR for CRCV. Of these 42 were from dogs with no respiratory signs (grade 1), 18 dogs had shown mild respiratory signs (grade 2), 46 had shown moderate (grade 3) and 13 severe respiratory signs (grades 4 and 5). Grades 4 and 5 were merged due to the low case numbers in these groups.

Table 4 shows the PCR results for coronavirus in dogs with different grades of respiratory disease. Specifically, Table 4 shows the RT-PCR results from tracheal and lung samples of 119 dogs with different respiratory signs (none to severe) using a nested PCR directed against the coronavirus spike gene as well as the number of positive samples out of total sample number and the percentage of positive samples (in brackets).

Table 4: RT-PCR results for tracheal and lung samples

Respiratory signs	Trachea: Positive samples	Lung Positive samples	Trachea and lung Positive samples
None	11/42 (26.2%)	8/42 (19.1%)	2/42
Mild	10/18 (55.6%)	4/18 (22.2%)	4/18
Moderate	9/46 (19.6%)	8/46 (17.4%)	2/46
Severe	2/13 (15.4%)	0/13	0/13

Establishment of a serological assay for CRCV

Because of the homology of the spike cDNA of CRCV to the spike region
 5 of bovine coronavirus, an ELISA antigen for BCV was used for serological
 analysis of CRCV.

Sera from five dogs with no history of infectious respiratory disease that
 had not been housed in the investigated kennel were tested. The OD values
 ranged from -0.013 to 0.39 with an average OD value of 0.154.
 10 Furthermore, sera from 30 dogs admitted to a veterinary clinic for various
 reasons were tested for antibodies to coronavirus. Of these, 20 samples
 showed an OD of <0.4 (-0.46 to 0.396) and 10 samples showed an OD of
 >1.0 (1.012 to 1.949). Samples with an OD of 0.6 or above were
 subsequently considered positive.

Comparison of the immune response to CRCV of dogs with and without respiratory disease

The BCV-antigen ELISA was performed using paired sera of 111 dogs from the study kennel. Of these, 81 dogs had shown symptoms of respiratory disease during a period of 21 days and 30 had remained healthy.

Of the group of dogs with respiratory disease, 17 were positive for antibodies to CRCV on the day of entry into the kennel and 64 were negative.

Of the 64 dogs with no detectable antibodies to BCV on day one, 63 tested positive on day 21. All 46 dogs out of these 63 for which a sample on day 7 was available tested negative on day 7. Therefore 63 dogs showed a seroconversion during the study-period whereas only one dog remained negative.

Of the 31 dogs that had remained healthy, 17 had antibodies to CRCV on the day of entry. All of the 13 dogs that were negative on day 1 tested negative on day 7 but showed a seroconversion by day 21.

Thus, of 34 dogs that were positive for antibodies to CRCV on arrival in the kennel, 17 developed respiratory disease (50%) whereas of 77 dogs that were negative on arrival, 64 developed respiratory signs during the study-period (83.1%), (Figure 12).

Therefore dogs that had no antibodies to CRCV on entry into the kennel had an increased probability of developing respiratory disease ($p < 0.001$).

Only one out of the 77 dogs that were negative on arrival remained negative during the study period of 21 days whereas 76 dogs showed a seroconversion.

Serology using canine enteric coronavirus (CECV) antigen

An ELISA assay using a canine coronavirus antigen was performed to investigate whether CRCV showed a serological cross reaction to canine enteric coronavirus. Sera from 27 dogs, previously tested for antibodies to
5 CRCV using the BCV antigen were selected.

It was found that eight dogs had antibodies to CECV on the day of entry into the kennel, of these four also had antibodies to CRCV. Nineteen dogs were found to be negative for CECV on day 1, 17 of these were also negative for CRCV. Of the 19 negative dogs, five showed a seroconversion
10 to CECV during the 21-day period of the investigation and 17 showed a seroconversion to CRCV.

Analysis of the prevalence of respiratory disease in this group showed that six out of the eight dogs (75%) that were positive for antibodies to CECV on day 1 developed respiratory disease. Out of the group of 19 dogs that had
15 no detectable antibodies to CECV on day 1, 15 showed signs of respiratory disease (78.9%), ($p=0.594$).

Virus isolation

Tracheal tissue samples from dogs that are identified as positive for CRCV RNA by RT-PCR are inoculated on cell cultures of canine adult lung
20 fibroblasts and MDCK cells. For some samples, virus isolation is also performed on A72 cells. The cultures show no signs of a cytopathic effect during three passages. After several passage, RNA is extracted from the cultures and tested for the presence of CRCV RNA by RT-PCR.

Discussion

This study reports the detection of a novel coronavirus, CRCV, in kennelled dogs with respiratory disease.

Coronaviruses have been reported to cause respiratory disease of man,
5 cattle, swine and poultry, but their presence in the respiratory tract of dogs and a possible association with canine infectious respiratory disease (CIRD) has not been determined.

Dogs were investigated from a kennel in which CIRD was endemic and could not be controlled by the use of vaccines recommended against CIRD.
10 Samples taken from the respiratory tract of these dogs were examined using RT-PCR primers directed to the conserved polymerase gene of coronaviruses (Stephensen *et al.*, 1999).

Initially, seven tracheal samples were found to be positive; the sequence of the RT-PCR products was determined and compared to all available
15 coronavirus polymerase gene sequences. This analysis revealed that the cDNA sequence obtained from the canine samples had the highest similarity to the polymerase gene of bovine coronavirus (98.8%) and human coronavirus OC43 (98.4%) but only a very low similarity to the polymerase gene of the enteric canine coronavirus (strain 1-71, 68.53% similarity).

20 A phylogenetic analysis was performed using the polymerase sequences of eleven additional coronaviruses. The coronavirus detected in the respiratory tract of dogs (CRCV) was located on a common branch with three group 2 viruses: BCV, HCV strain OC43 and HEV. However, canine enteric coronavirus, a group 1 coronavirus, was shown to be only distantly related.

Canine respiratory coronavirus therefore is a novel coronavirus of dogs that is most closely related to BCV and HCV-OC43, both of which are known to cause respiratory disease.

To obtain more sequence information and to further determine the relationship to other coronaviruses using a more variable gene, a part of the spike gene was analysed. Since CRCV had been shown to be most similar, to BCV and HCV-OC43, an alignment of the sequences of their spike genes was used to design a nested set of primers. Nested primers were chosen to achieve a more sensitive assay.

10 Sequencing of the products of this RT-PCR confirmed the high similarity of CRCV with BCV and HCV-OC43.

The presence of antibodies to CRCV was analysed using an ELISA based on a BCV antigen because of the high sequence similarity of the two viruses in the spike cDNA. The ELISA results confirmed the presence of a virus similar to BCV in the study population.

The prevalence of antibodies was 30% at the time of entry into the kennel and 99% after 21 days.

Interestingly and unexpectedly, serological analysis revealed that dogs with antibodies to CRCV on day of entry into the kennel developed respiratory disease less frequently than dogs without antibodies ($p < 0.001$). Therefore the presence of antibodies to CRCV had a protective effect against respiratory disease in this population.

Almost all dogs negative on day of entry into the kennel showed a seroconversion to CRCV within three weeks, indicating that the virus is highly contagious. Serology using an antigen for canine enteric coronavirus (CECV) showed a much lower prevalence of antibodies to CECV on day

21. Therefore the BCoV-ELISA results did not reflect an infection with canine enteric coronavirus and the cross-reactivity between the two antigens seems to be low.

Serum antibodies to CRCV were present in about 30% of dogs of various origins including dogs entering a re-homing kennel as well as pet dogs. The presence of CRCV is therefore not limited to the investigated kennel and the virus seems to be established in the dog population.

By PCR, CRCV was detected in tracheal tissue and lung tissue and therefore appears to infect the upper and lower respiratory tract of dogs. Within the kennelled population, CRCV-RNA was detected in 27.3% of dogs with all grades of respiratory disease as well as in 26.2% of dogs that were apparently healthy at the time of euthanasia.

CRCV-RNA was most frequently found in the trachea of dogs with mild cough (55%). Studies using the human coronavirus strain 229E have shown, that coronaviruses can cause disruption of the respiratory epithelium and ciliary dyskinesia (Chilvers *et al.*, 2001). Without being bound by theory, we believe that an infection with CRCV has a similar effect, and that the virus plays an important role in the early stages of the pathogenesis of CIRD. By damaging the respiratory epithelium and disrupting ciliary clearance CRCV facilitates the entry of other viral or bacterial pathogens. Therefore while CRCV infection on its own may cause only mild respiratory symptoms, in conjunction with other pathogenic agents it could lead to severe respiratory disease.

The pathogenesis of CIRD has not been thoroughly investigated since the 1970s when *Bordetella bronchiseptica*, canine adenovirus type 2 and canine parainfluenza were determined to be the main causes of the disease. However the vaccination of all dogs against CPiV, CAV-2 and distemper

virus did not help to control the disease in this kennel despite evidence that the majority of dogs responded to the vaccine within 21 days (data not shown).

This study shows an association of a novel canine respiratory coronavirus with CIRDC. The aetiology of CIRDC therefore needs to be re-evaluated and the role of novel microorganisms or microorganisms previously not associated with the disease has to be established.

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Example 2: Cloning and expression of CRCV Spike

The CRCV Spike gene was cloned using the primers listed in Table 5 and using the following cloning strategy, which is illustrated in Figure 18.

1. The spike gene was amplified in four overlapping fragments
5 (A,B,C,D).
2. The PCR product Sp5-Sp2 (B) was joined to the product Sp1-Sp8 (C) using the *PvuII* site in the overlap.
3. This fragment was cloned into the pT7blue2 vector (Novagen) using the restriction sites *NcoI* and *BstXI*.
- 10 4. The PCR fragment SpFXho-Sp6 (A) was joined to BC using the restriction site *BstXI* in the overlap and the *XhoI* site that had been incorporated into the primer SpF-Xho.
5. Fragment ABC was moved into the baculovirus transfer vector pMelBacB (Invitrogen) using the restriction sites *XhoI* and *NcoI*.
- 15 6. The PCR fragment Sp7-SpR-HisTag- Eco (D) was joined to ABC using the restriction site *NcoI* in the overlap and the *EcoRI* site that had been incorporated into the primer SpR-Eco-HisTag resulting in the complete spike gene in pMelBacB (Spike MelBac). This construct contains a HisTag (6xHis) at the C terminus of the expressed protein.
- 20 7. For mammalian expression the complete gene was moved to pSecTagA (Invitrogen) using the *BamHI* site in pMelBacB and the *EcoRI* site at the end of ABCD resulting in the plasmid SpikeSecTag.

Construction of a recombinant baculovirus

A co-transfection was performed in Sf9 cells using the Bac-N-Blue baculovirus DNA (Invitrogen) and Spike MelBac. The resulting baculovirus (AcSpCRCV 1-11) was shown to contain a full-length insert by
5 PCR using primers (Invitrogen) located upstream and downstream of the recombination site.

Expression in mammalian cells

The plasmid Spike SecTag was transfected into BHK-21 cells using Lipofectamine (Invitrogen). Expression of the Spike protein was analysed
10 using a serum sample from a dog that had been shown to be positive for antibodies to CRCV using ELISA (BCV antigen obtained from Churchill) and a positive control serum for BCV obtained from Churchill (chicken anti BCV). The transfected cells showed a positive signal in an immunofluorescence assay using the canine or the chicken serum and a
15 FITC labelled conjugate (FITC anti-dog IgG or FITC anti Chicken IgG).

Table 5: Primers designed from an alignment of the spike genes of bovine coronavirus (GenBank accession No. AF058942) and human coronavirus, OC43 (GenBank accession No. L14643)

Name	Sequence	SEQ ID NO:	Location in BCV spike gene
Sp 1	5'-CTT-ATA-AGT-GCC-CCC-AAA-CTA-AAT	40	1637-1660
Sp 2	5'-CCT-ACT-GTG-AGA-TCA-CAT-GTT-TG	41	2258-2236
Sp 3	5'-GTT-GGC-ATA-GGT-GAG-CAC-TG	42	1666-1686
Sp 4	5'-GCA-ATG-CTG-GTT-CGG-AAG-AG	43	2107-2088
Sp 5	5'-AAC-GGT-TAC-ACT-GTT-CAG-CC	44	931-950
Sp 6	5'-CAA-GTA-AAT-GAG-TCT-GCC-TG	45	1121-1102
Sp 7	5'-GGC-TGC-CAC-CTC-TGC-TAG-TC	46	2919-2938
Sp 8	5'-ATT-GTT-AAA-TGC-ATT-AGC-AAT-AAG-C	47	3069-3045
SpF	5'-TTT-ITG-ATA-CTT-TTA-ATT-TCC-TTA-CC	48	4-29
SpR	5'-GTC-GTC-ATG-TGA-WGT-TTT-RAT-TAC	49	4089-4066
SpF- <i>Xho</i> I	5'-AGC-TCG-AGC-TTT-TTG-ATA-CTT-TTA-ATT-TCC-TTA-CC	50	
SpR His- <i>Eco</i> RI	5'-TTG-AAT-TCT-TAA-TGA-TGA-TGA-TGA-TGA-TGG-TCG-TCA-TGT-GAW-GTT-TTR-ATT-AC	51	

SpF-*Xho*I contains a *Xho* I site (bold). SpR-His-*Eco*RI contains a 6xHisTag (double-underlined), a stop codon (underlined) and an *Eco*RI site (bold)